Synthesis and In Vitro Pharmacological Evaluation of Novel Ligands for Histamine Receptors

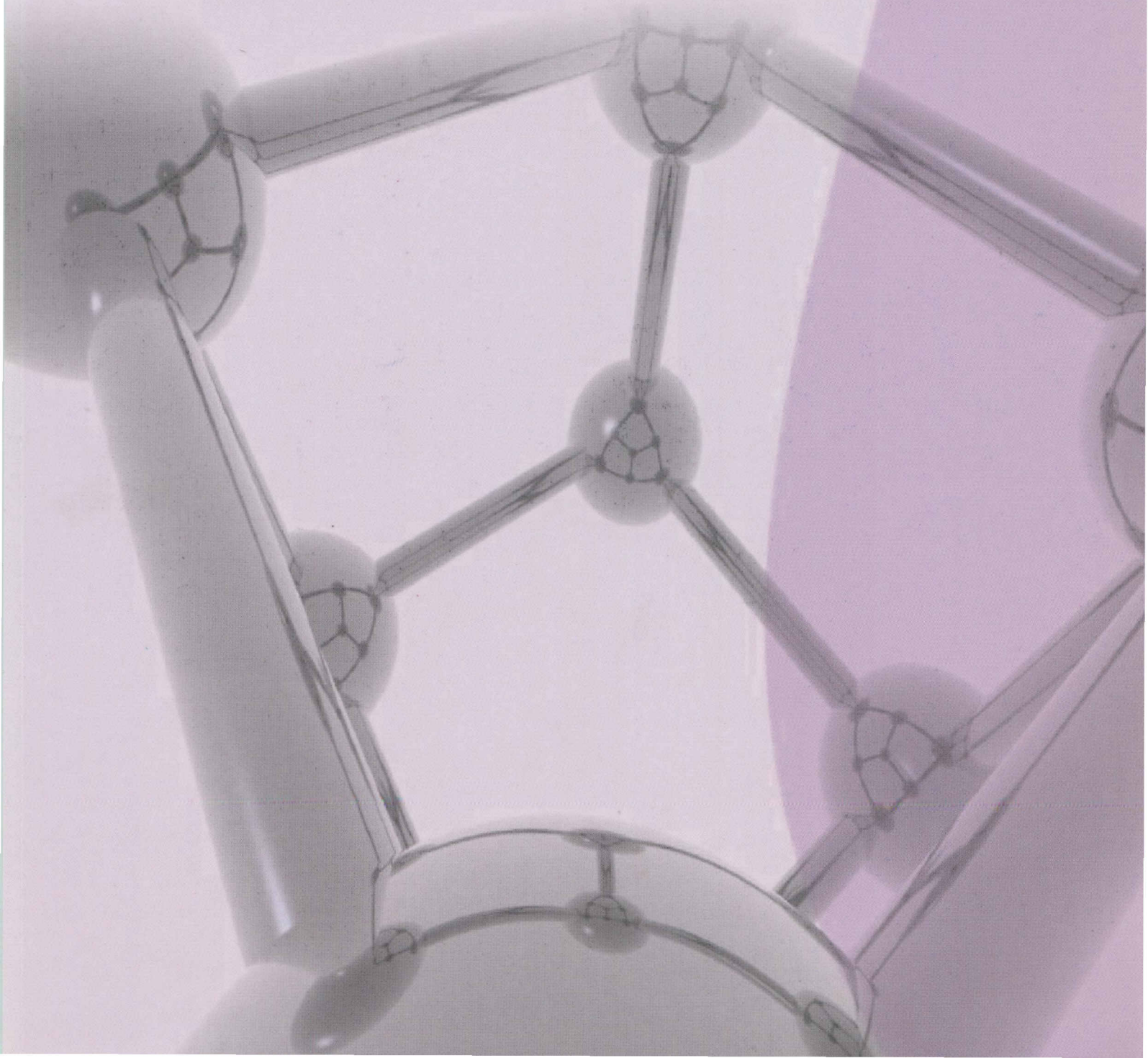
Govoni, M.

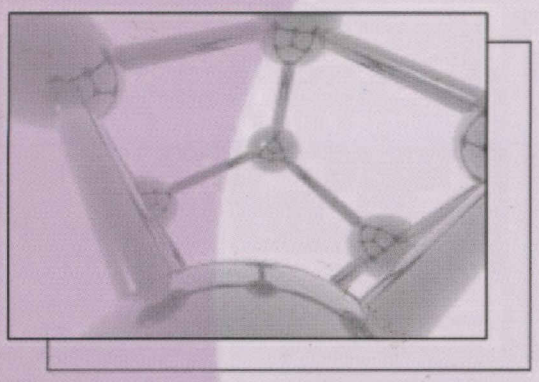
2005

document version
Publisher's PDF, also known as Version of record

Synthesis and *In Vitro* Pharmacological Evaluation of Novel Ligands for Histamine Receptors

Marinella Govoni





Synthesis and *In Vitro* Pharmacological Evaluation of Novel Ligands for Histamine Receptors

Marinella Govoni

The research described in this thesis was performed at the Leiden/Amsterdam Center for Drug Research, Division of Medicinal Chemistry, Department of Pharmacochemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

Part of the work described in Chapter 7 was performed at the School of Pharmaceutical Sciences, University Park, Nottingham NG7 2RD, and Institute of Cell Signalling, Medical School, Queen's Medical Center, Nottingham NG7 2UH, University of Nottingham, UK.

VRIJE UNIVERSITEIT

Synthesis and *In Vitro* Pharmacological Evaluation of Novel Ligands for Histamine Receptors

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Exacte Wetenschappen
op donderdag 23 juni 2005 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Marinella Govoni

geboren te Cento, Italië

promotoren: prof.dr. R. Leurs
prof.dr. H. Timmerman

Ai miei genitori

To my parents

Table of contents

Chapter 1

Introduction

Contents

1.1 G-protein coupled receptors (GPCRs)

The superfamily of GPCRs has more than 1000 members and is the largest family of proteins in the body. GPCRs mediate signalling of stimuli as diverse as light, ions, small molecules, peptides and proteins and are the targets for many pharmaceuticals. The term "GPCR" originates from the ability of these receptors to associate with heterotrimeric G-proteins and through them stimulate or inhibit the formation of second messengers such as inositol-3,4,5-tris-phosphate (IP_3) and cyclic AMP or modulate the function of ion channels.[1,2] The GPCRs do not share a large overall amino acid sequence identity and their topologies vary considerably. The only structural feature shared by all GPCRs is the presence of seven predicted hydrophobic transmembrane α-helical segments of 20-25 amino acid residues.[1,3] GPCRs are therefore also frequently termed "7-TM receptors" (Figure 1).

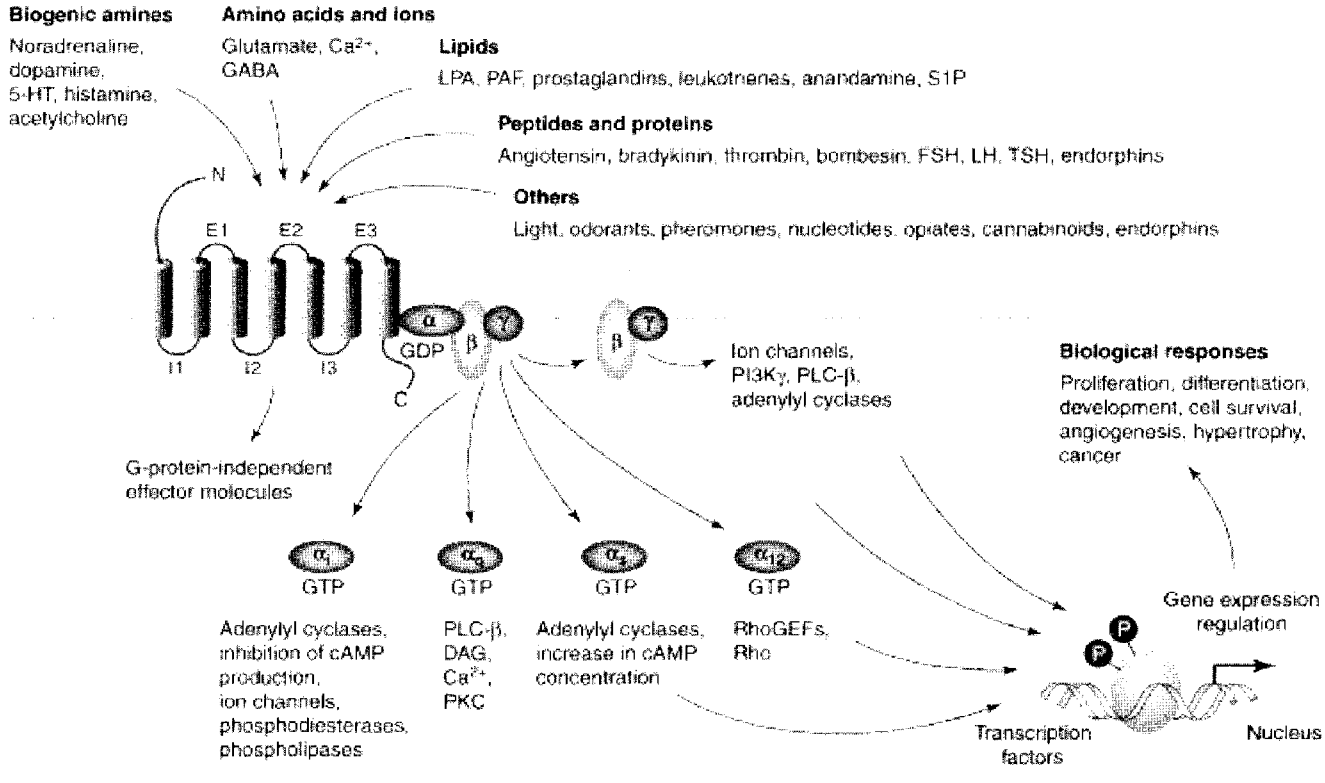


Figure 1. Diversity of GPCRs-mediated signalling. A wide variety of ligands, including biogenic amines, amino acids, ions, lipids, peptides and proteins, use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and -independent pathways. Such signalling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Abbreviations: DAG, diacylglycerol; FSH, follicle-stimulating hormone; GEF, guanine nucleotide exchange factor; LH, luteinizing hormone; LPA, lysophosphatidic acid; PAF, platelet-activating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone (adapted from Marinissen *et al.*[260]).

1.1.1 Constitutive GPCR signalling and reclassification of GPCR ligands

In the late 1980s and early 1990s, observations of constitutive activity of GPCR (assumed to correspond to a pre-coupling to the G-protein in the absence of agonist) and inverse agonism (suppression of constitutive activity by inverse agonists) fundamentally changed the perception of the molecular mechanisms underlying GPCR function. Originally reported for the δ-opioid receptor in NG108-15 cells,[4] this constitutive GPCR activity has now been described for a variety of GPCRs.[5-7] Since this class of receptors is the target of a large fraction of drugs currently used in therapeutics,[8] the concept is of obvious practical importance.

Several model have been proposed to account for this "revised" pharmacology. The extended ternary complex model or "two-state" model was formulated to explain these observations (Figure 2).[9] A simpler version of this model was published in 1995 by Leff (Figure 2).[10]

A common feature of all the proposed models is the assumption of existence of at least two conformers of the receptor, one active (coupled, R*) and the other one inactive (uncoupled, R). The R* state is considered to be responsible for effective G-protein activation and the equilibrium between R and R* determines the level of basal, constitutive GPCR signalling.[5,6,11]

An agonist is a molecule that binds to and stabilises or increases the fraction of the active state of a GPCR (R*) so that it can interact with and activate a G-protein. Full agonists, displaying an intrinsic activity (α) of 1, maximally stimulate the protein; partial agonists can only activate the GPCR to a certain degree (Figure 3). The actions of agonists can be prevented by so-called antagonists. Originally, these ligands were thought to compete with the agonists for the same binding site but not to affect the GPCR activity directly. With the notion of spontaneous GPCR activity, it has become clear that antagonists should be reclassified. So-called inverse agonists antagonize the actions of agonists, but can also reduce the basal GPCR activity themselves by stabilizing or enriching the inactive state (R). In the same way as agonists can exhibit a spectrum of activity from low-intrinsic-activity partial agonists to full agonists (α=1), a full spectrum of (negative) intrinsic activity (α) from −1 to 0 can be observed (Figure 3).[5,6]

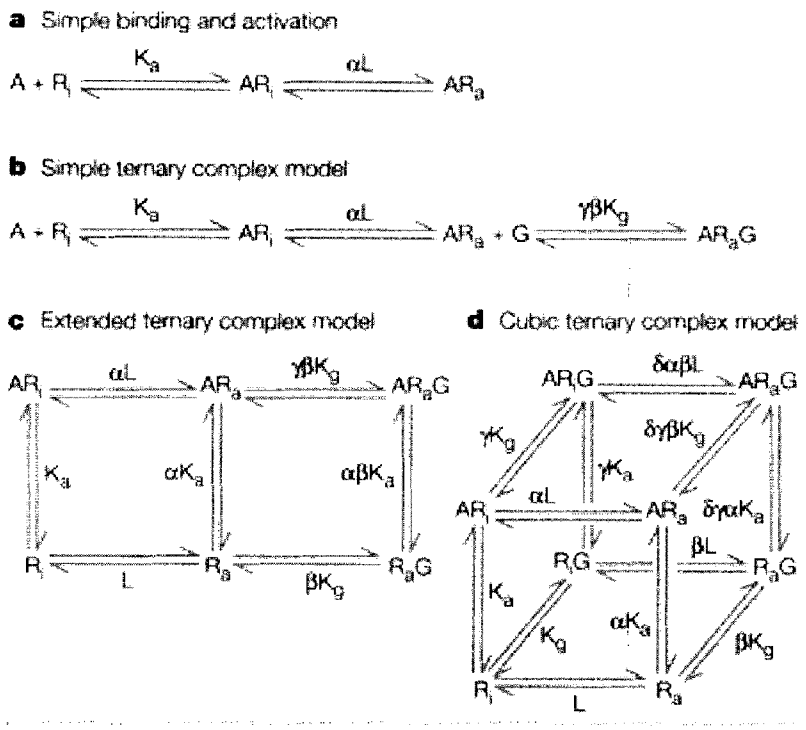


Figure 2. Commonly used models of GPCR system. The classical view of receptor activation describes agonist (A) binding to an inactive receptor (R) to form a complex (AR) that, because of the efficacy of the agonist, isomerizes the receptor to the active state (AR*) (panel a). Following the discovery of ternary complexation for GPCRs, it was proposed that the activation of the receptor was followed by topographically distinct binding of the active-state receptor to the G protein (G). Response then emanates from the ternary AR*G complex (panel b). The extended ternary complex model[9] (panel c) allows for the spontaneous formation of an active-state receptor (R*) independent of the presence of an agonist. R* can then interact with, and activate, a G protein. Activation of the receptor (from R to R*), either spontaneously or through ligand binding, modifies the affinity of the receptor for the G protein by the factors β and γ. It is this modified affinity of the receptor for the G protein that is the expression of efficacy. The cubic ternary complex (CTC) model[261] (panel d) does the same, but also allows the inactive-state receptor (R) to form a non-signalling complex with the G protein, with the affinity of the receptor for the G protein being modified by the factors δ, β and γ. This non-signalling complex is required thermodynamically as a feature of the complete system, but might not be physiologically relevant (adapted from Kenakin[262]).

Compounds that bind to GPCRs without altering the equilibrium between active and inactive states of the receptor are termed "neutral antagonists" ($\alpha=0$) and are rather rare. These are ligands that have no capacity to regulate basal transduction when they bind to a GPCR but compete with both agonists and inverse agonists for the binding site and by doing so reverse the function of both classes of agonists (Figure 3).

Hence, receptor ligands should be considered as triggering a continuum of effects from full agonists to neutral antagonists and up to full inverse agonists.

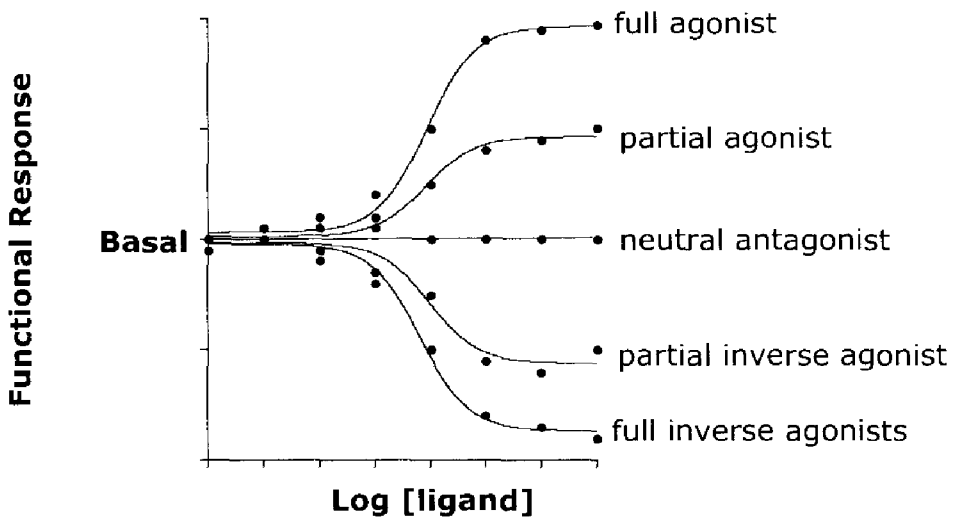


Figure 3. Example of functional responses of agonists and inverse agonists in a receptor system that displays constitutive receptor activity. Full agonists induce the maximum response, whereas full inverse agonists induce the full inhibition of the basal response that is the result of agonist-independent receptor signalling (constitutive activity). Neutral antagonists have no functional response, while partial agonists and partial inverse agonists result in partial responses.

1.1.2 Inverse agonism and neutral antagonism at GPCRs

Inverse agonism was originally described for ionotropic receptors such as the $GABA_A$ receptor,[12] but has subsequently been described extensively for a wide range of both native and mutated GPCRs.[6,13-16]

Full and partial inverse agonists have been described in several systems, e.g. α_{1A}-, α_{1B}-, α_{2A}- and β_2-adrenoceptors, as well as 5-HT_{1A} receptors, and only a few drugs possess no efficacy, with only a few neutral antagonist having been recognized.[17-20] In fact, a large fraction of compounds previously known as antagonists have been recognized to act indeed as inverse agonists on relevant systems. Neutral antagonists display equal affinity for R and R*, a condition obviously difficult to fulfil, which explains the low number of such compounds that could be identified. Some examples of drugs with important therapeutic actions that have been shown to be inverse agonists include cimetidine (which acts on histamine H_2 receptors, Figure 6B. See also section 1.2.3.3),[21] haloperidol (which acts on dopamine D_2 receptors),[22] prazosin (which acts on α_1-adrenoceptors),[17] timolol (which acts on β_2-adrenoceptors),[19] clozapine (which acts on D_2 receptors and 5-HT_{2C} receptors),[22,23] and several antihistamines, such as cetirizine, epinastine, loratadine (which act on histamine H_1 receptors, Figure 5C. See also section 1.2.2.3).[24]

1.1.3 Physiological relevance of inverse agonism

It is essential to determine whether this newly identified inverse agonism matters in terms of drug effects in human or animals. Several descriptions of inverse agonism rely on effects in recombinant systems but it is of course important to examine if comparable effects are seen in native tissues as well.

Acute administration of inverse agonists to native tissues might be expected to suppress the basal (agonist-independent) activity of a system, which of course depends on that native system exhibiting agonist-independent activation. Thus, a key question for the potential importance of inverse agonists and whether they may or not have inherent benefits as therapeutics agents relates to the level of constitutive activity of the specific GPCRs. Constitutive receptor signalling of wild-type receptors has been demonstrated repeatedly,[4,6,25] but again it was described in artificial systems, so that the physiological relevance of the process has been a matter of debate. As such, a thorny issue is whether significant levels of constitutive activity are seen only in recombinant systems in which GPCR expression levels can be easily manipulated and are relatively high. Early data already suggested that this is not the explanation in all cases,[16] but only recently a clear example of *in vivo* evidence of constitutive receptor activity (that was not due to overexpression of receptors) has been provided for the histamine H_3 receptor.[26]

Alternatively, inverse agonists might require chronic administration to exert their effects. There are many examples of the chronic administration of drugs that were considered to be antagonists leading to an upregulation of receptors,[14] which may render such compounds unfavourable as therapeutics. A number of wild-type GPCRs that display significant levels of constitutive activity, such as the β_2-adrenoceptor and the histamine H_2 receptor and which have been classical targets for clinical intervention via receptor "blocker" strategies have been shown to have the potential to be upregulated via sustained treatment with inverse agonist ligands. This receptor upregulation could have important physiological implications. For example, for the H_2R, the upregulation produced by the inverse agonists is not desirable and may be a factor in the observed clinical tolerance to the drugs described as inverse agonists.[27,28] Indeed, it has been demonstrated that prolonged treatment of patients with H_2R inverse agonists such as cimetidine leads to the development of tolerance and an increased H_2R sensitivity following withdrawal.[21,29] Furthermore, treatment of patients with β-blocker drugs (many of which may actually display inverse agonist properties) is known to produce a degree of β-adrenoceptor upregulation.[30] More examples of GPCRs undergoing receptor upregulation in vivo after "antagonist treatment" have been reported.[31-33] Clearly, neutral antagonists for those GPCRs would be valuable pharmacological tools to study the potential (patho)physiological role of constitutive GPCR activity.

For GPCRs with high constitutive activity, an intriguing idea is that endogenous antagonists/inverse agonists are produced to dampen this activity. For example, the agouti-related protein (AgRP) is an endogenous ligand of the melanocortin MC_3 and MC_4 receptors that is involved in the regulation of body weight and its level of expression is adjusted to maintain energy balance. In addition to antagonizing the effects of the melanocortin receptor agonist α-melanocyte-stimulating hormone (α-MSH), AgRP suppresses the constitutive activity of melanocortin MC_3 and MC_4 receptors, which characterizes AgRP as an inverse agonist rather than a neutral antagonist. AgRP and smaller fragments have thus been classified as endogenously produced inverse agonists.[34-36]

If the regulation of constitutive receptor activity *in vivo* is important, more endogenous inverse agonists might exist. Probably, the best-known natural inverse agonist is retinal.[37] Some natural ligands for chemokine receptors are also reported to be inverse agonists,[38-40] but the number of identified endogenous inverse agonists remains so far limited.

1.1.4 (Patho)physiological importance of GPCRs constitutive activity

The structural features of the GPCR that determine the level of constitutive activity are poorly understood. Important informations have however been obtained from studies with so-called constitutively active mutant (CAM) receptors. Site directed mutagenesis of GPCRs in regions governing the interaction with G proteins, first shown for the β_2-adrenergic receptor mutated at the level of the third intracellular loop,[9] results in constitutive activation.[41] The CAM-GPCRs have not only proved to be helpful experimental tools, but also to be of physiological relevance.

Agonist-independent activity of heptahelical receptors following pathological mutations in the gene encoding the corresponding GPCR was shown to be the molecular basis for various human diseases, such as familial male precocious puberty (LH receptor),[42] retinal degeneration (rhodopsin),[43] familial hyperthyroidism (TSH receptor)[44] and hyper-pigmentation (MSH receptor).[45] It is obvious that for these genetic disorders inverse agonists are essential for silencing the mutant GPCRs, as neutral antagonists would be of no use.

However, compounds with close to zero intrinsic activity (i.e. true neutral antagonists) are vital pharmacological tools in ligand classification because any compound with this characteristic that binds in a competitive manner will act as a functional antagonists to compounds with agonist or inverse agonist properties. Neutral antagonists can thus be used to exclude apparent inverse agonism that derives from competition with an endogenous ligand rather than via suppression of receptor constitutive activity.

However, some care must be exercised in the use of neutral antagonists and subsequently in the design of new drugs acting as neutral antagonists. Bearing in mind the previously

discussed existence of endogenous inverse agonists as a control mechanism to fine-tune physiological processes, e.g. AgRP (see also section 1.1.3),[36] it is evident that neutral antagonists might not only antagonize the effects of endogenous agonists but also of (putative) endogenous inverse agonists. Consequently, the latter effect might result in neutral antagonists producing agonist effects *in vivo*.

Despite the large number of studies carried out on inverse agonism and neutral antagonism at GPCRs, it remains unclear whether inverse agonists offer inherent advantages over neutral antagonists in the vast majority of clinical scenarios, or indeed whether the inverse agonism of some medicines contributes significantly to their effectiveness. Unravelling this issue remains a key challenge and simple heterologous expression systems may not provide a sufficient assessment of the significance of inverse agonism in a clinical setting.

1.2 The histamine receptors

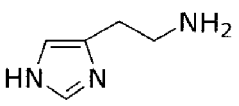
Members of the histamine receptors family have played an important role in the recognition of the importance of constitutive GPCR signalling, including the potential drawbacks in the use of inverse agonists as therapeutics,[21] and the occurrence of constitutive activity *in vivo*.[26]

In the next sections, the four identified histamine receptor subtypes, their distribution, function, signal transduction mechanism and constitutive activity, as well as the various specific ligands for each receptor that can be used as pharmacological tools will be briefly reviewed.

A short review on the use of fluorescent ligands for the study of receptors and a perspective on the currently available fluorescent ligands for the histamine receptors will be given as well.

1.2.1 Biological actions of histamine

Since its first pharmacological description as an endogenous substance in 1910,[46,47] the biogenic monoamine histamine (Figure 4) has proven to exert tremendous influence over a variety of (patho)physiological processes.



Histamine

Figure 4. Structure of histamine.

Nowadays, it is well established that histamine mediates its actions through histamine receptors, of which four subtypes (H_1, H_2, H_3 and H_4) are currently recognized and for which the corresponding genes have been cloned.[48-51] All four histamine receptors belong to the superfamily of GPCRs.

Initially, research in the histamine field was mainly focussing on the role of histamine in allergic diseases and this intensive research resulted in the development of several potent "antihistamines" (e.g. mepyramine, also known as pyrilamine, Figure 5C), which were useful in inhibiting certain symptoms of allergic conditions.[52] The observation that these "antihistamines", now recognized as inverse H_1R agonists, could not block the histamine-induced effects on heart and stomach, led Ash and Schild in 1966 to the hypothesis that histamine should act via at least two distinct receptor subtypes.[53] This hypothesis became generally accepted in 1972 when Black *et al.* succeeded in the synthesis of a series of new compounds (e.g. burimamide, Figure 6C), which were able to block the effects of histamine on the stomach and the heart.[54] These H_2R antagonists proved to be very useful in the therapy of gastric ulcers. The identification, in 1983, by Arrang *et al.* of a new histamine receptor subtype (H_3R), a presynaptic receptor,[55] gave rise to a new field of interest. The cloning of the H_3R gene in 1999[50] confirmed the existence of this receptor at the molecular level and the H_3R is now considered a general regulatory system in the CNS and a potential target for new therapeutics.[56,57] The existence of a fourth histamine receptor was postulated in 1994 by Raible and co-workers[58] and confirmed by the recent cloning of the H_4R gene by several groups.[51,59-63] In view of its expression pattern the H_4R has been suggested as a new target for the regulation of hematopoietic and immune functions.

1.2.2 Histamine H_1 Receptor

1.2.2.1 Distribution, function and signal transduction mechanisms

The H_1R is distributed in a wide variety of tissues, including mammalian brain, smooth muscle from airways, gastrointestinal tract, genitourinary and cardiovascular systems, adrenal medulla, endothelial cells and lymphocytes.[47,64,65] Some of the responses elicited upon stimulation of the H_1R are smooth muscle contraction, stimulation of NO formation, endothelial cell contraction, increased vascular permeability, stimulation of hormone release, negative inotropism, depolarisation (block of leak potassium current) and increased neuronal firing. Some of these effects are manifest in the so-called "triple response": redness, itch and swelling of allergic response, in fact the H_1R plays a major role in allergic conditions.

The bovine adrenal medulla H_1R gene was cloned in 1991 by expression cloning in the *Xenopus* oocyte system.[48] The availability of the bovine sequence and lack of introns has enabled the

H_1R to be cloned from several species, including rat,[66] guinea pig,[67,68] mouse[69] and human.[21,70-72] The homology between the several encoded proteins is very high in the intracellular domains (±90%), but is significantly lower in the intracellular and extracellular parts. No receptor heterogeneity has been found so far, although there exist some slight pharmacological differences among the receptor proteins.[48,68,70]

The primary mechanism by which H_1Rs produce functional responses in cells is the activation of phospholipase C (PLC) via a pertussin toxin-insensitive G-protein related to the $G_{q/11}$ family of G-proteins,[64,73] resulting in formation of inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate hydrolysis, which ultimately leads to an increase in the intracellular calcium concentration.[52,64,74,75] In addition to effects on the inositol phospholipid signalling systems, H_1R stimulation can lead to the activation of several other signalling pathways, many of which appear to be secondary to changes in intracellular calcium concentration or the activation of protein kinase C (PKC). Thus, histamine can stimulate nitric oxide synthetase (NOS) activity,[76,77] phospholipase A_2 (PLA_2) activity,[78,79] with liberation of arachidonic acid from cell membrane, and changes in the intracellular levels of cAMP.[80,81] Furthermore, stimulation of the H_1R was recently shown to promote transcription of genes under the control of nuclear factor kappa B (NF-κB).[82-84] As both the H_1R and NF-κB are known to be involved in allergic conditions and inflammatory response,[85,86] it is attractive to speculate that in such disorders the coupling of the H_1R to the NF-κB pathway is of pathophysiological importance.

1.2.2.2 Constitutive H_1R signalling and reclassification of H_1R antagonists

The human H_1R has recently been shown to be constitutively active.[24] Transient expression of the wild-type human H_1R in COS-7 cells led to an expression-dependent increase in luciferase activity in a H_1R-dependent reporter-gene assay. This constitutive signalling was inhibited potently and completely by the H_1R antagonist mepyramine (Figure 5C), indicating that mepyramine in fact acts as an inverse agonist at the human H_1R. In contrast, neither H_2R nor H_3R antagonists affected this response.

H_1-antihistamines are widely used to relieve the symptoms of allergic reactions and have become one of the safest and most prescribed drug families worldwide. Constitutive H_1R-mediated NF-κB activation has been shown to be inhibited by all the clinically used H_1-antihistamines tested to date, including cetirizine (Zyrtec®), epinastine (Flurinol®) and loratadine (Claritin®) (Figure 5C), indicating that all of these drugs are inverse H_1R agonists[24,84] and thus shedding new light on their presumed mechanism of action at the H_1R.

At present, no data on the (patho)physiological relevance of the constitutive H_1R activity are yet available. It is nevertheless tempting to speculate that constitutive H_1R activity may contribute to pathophysiological conditions where either H_1R upregulation occurs, such as in

patients with allergic rhinitis,[87], in vascular smooth muscles in atherosclerosis[88] and in specific brain areas upon seizures,[89,90] or $G\alpha_q$ protein levels are found to be elevated, such as in the guinea pig nasal mucosa in a model of nasal hyperresponsiveness.[91] The discovery of inverse agonism has also raised the general question of potential differences in therapeutic outcome upon treatment with inverse agonists or neutral antagonists. Although an inverse H_1R agonist would suppress any apparent constitutive H_1R activity, long-term exposure of cells expressing constitutively active GPCRs to inverse agonists may result in receptor upregulation,[14,21,30,31,33] a phenomenon that could have clinical significance. Upregulation of endogenously expressed H_1Rs and increased effectiveness of histamine to induce the formation of inositol phosphates upon inverse H_1R agonists treatment have indeed been recently described,[92] underscoring the potential benefit of neutral antagonists. However, no data on the (patho)physiological importance of H_1R upregulation are available to date.

1.2.2.3 H_1R specific ligands

In contrast to the well-defined clinical utility of H_1R-antihistamines, the therapeutic value of H_1R agonism is still unclear. Betahistine, a H_1R agonist with micromolar potency that also exhibits H_3R antagonistic properties,[93] has been used in the treatment of Ménière's disease, but the therapeutic action has not been clearly linked to its H_1R agonism. Activation of H_1Rs may play a role in seizures,[94,95] where H_1R agonists may act as anticonvulsants.[96,97] In additions, there is increasing evidence of H_1R involvement in arousal, cognition and memory.[98-100]

However, so far H_1R agonists have attracted attention only as pharmacological tools for fundamental research of the function of H_1Rs in several (patho)physiological conditions, and, importantly, for deciphering the enigmatic molecular mechanism of H_1R activation. Many efforts have been done toward the identification of highly potent and selective H_1R agonists.

Modification of the imidazole ring of histamine has shown to be the most promising approach to develop potent and selective H_1R agonists. Thus, replacement of the imidazole ring of histamine by 2-thiazolyl or 2-pyridiyl groups yields compounds (2-(2-thiazolylethyl)amine (TEA) and 2-(2-Pyridylethyl)amine (PEA), Figure 5A) with selectivity for the H_1R, which also highlight that the presence of the tautomeric N^{π}-N^{τ} system of the imidazole ring is not obligatory.[75,101,102] Substitutions in the 2-position of the imidazole ring of histamine have produced compounds that are among the most selective H_1R agonists available,[103] e.g. 2(3-bromophenyl)histamine and 2[3-(trifluoromethyl)-phenyl]histamine (Figure 5A).

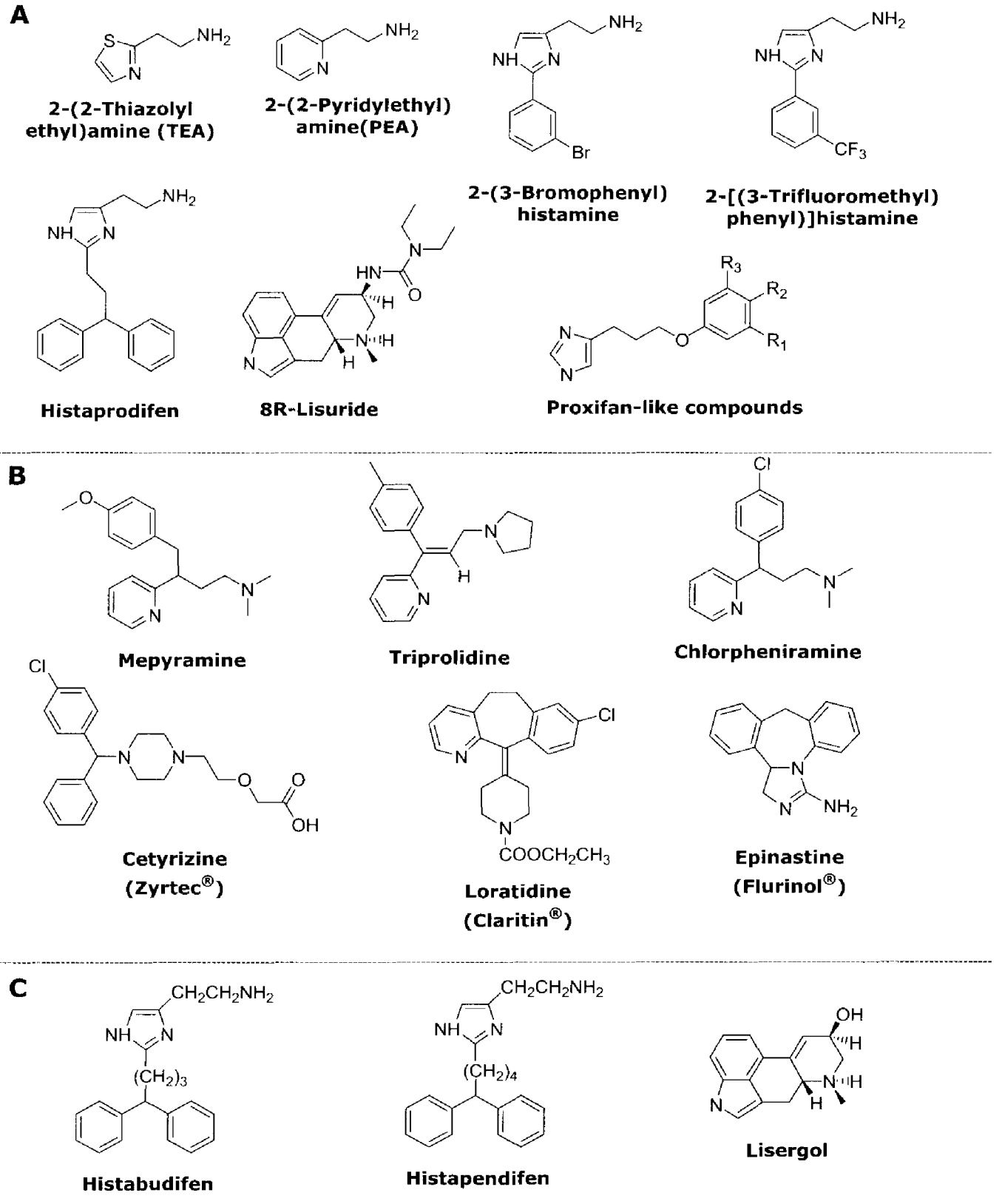


Figure 5. Structures of several H_1R ligands. A, H_1R agonists. B, Inverse H_1R agonists. C, Neutral H_1R antagonists.

Recently, Schunack *et al.* introduced a new class of highly active H_1R agonists, the so-called "histaprodifens", which are clearly more potent than histamine. Histaprodifen (Figure 5A) and its N^{α} mono- and di-methylated derivatives have been identified as highly potent and selective H_1R agonists.[104,105] These compounds combine a histamine moiety with a ω,ω-diphenylalkyl substituent, a characteristic feature of the H_1R antagonist pharmacophore, and thus they interact with both agonist and antagonist binding site of the H_1R.[106] However, several histaprodifens have been shown to possess species selectivity towards the guinea pig H_1R over the human H_1R, which may be explained by higher conformational flexibility of gpH_1R relative to hH_1R.[107]

A few interesting partial H_1R agonists have been discovered very recently. For example, 8R-lisuride (Figure 5A) has been identified as a potent stereospecific H_1R partial agonist,[108] which also represents the most potent H_1R agonist known to date. Moreover, some H_3R antagonists belonging to the proxyfan series (Figure 5A) have been characterized in a functional *in vitro* assay using guinea pig ileum, where they unexpectedly showed partial H_1R agonism.[109] Those compounds lack a basic side chain and as such they represent a new and unique class of imidazole-type H_1R agonists.

The classification of the H_1R antagonists has recently been altered, in fact, as already mentioned in section 1.2.2.2, H_1Rs have been shown to exhibit constitutive activity, which in turn led to the reclassification of most H_1R antagonists into H_1R (partial) inverse agonists.[24]

Although a wide array of potent and selective H_1R inverse agonists are available to study the H_1R, one should always be aware of possible muscarinic and serotoninergic properties and local anaesthetic effects of many classical H_1R antagonists.[64,102] Currently, mepyramine, triprolidine and chlorpheniramine (Figure 5B) are mainly used as H_1R inverse agonists in pharmacological studies. Mepyramine, due to its high affinity and good receptor selectivity,[64,110] is the commonly used reference compound for studying the affinity of ligands to the H_1R. Mepyramine is rather selective if used at concentrations below 100 nM. Above this level, interactions with receptors other than the H_1R cannot be excluded.[64] The tritiated analogue of mepyramine, i.e. [^{3}H]-mepyramine, is also successfully used as an H_1R radioligand.[75,102]

During the last decades modern H_1-antihistamines have been developed with negligible or only moderate affinity for other receptors, like the cholinergic and serotoninergic receptors. A major breakthrough was achieved with the development of non-sedating H_1-antihistamines (second generation H_1-antihistamine), which appeared to have a strongly reduced potential to enter the CNS, e.g. astemizole,[111] cetirizine (Figure 5B),[112] terfenadine[113] and its active metabolite fexofenadine,[114] and loratadine (Figure 5B).[115] In fact, the widespread distribution of the H_1R in cerebral areas involved in wakefulness and cognition[116] presumably accounts for the sedative properties of first generation H_1-antihistamines. However, some of these second generation H_1-antihistamines, notably terfenadine and astemizole, have been associated with prolongation

of the QT interval and the development of torsades de pointes, a potentially fatal ventricular arrhythmia.[117,118] In contrast, studies in human volunteers showed the absence of electrocardiographic effects of azelastine, cetirizine, fexofenadine, and loratadine administered at several times the recommended dose or concomitantly with agents that inhibit their metabolism and elimination, thus indicating that the potential to cause ventricular arrhythmias is not a class effect of second-generation H_1-antihistamines and that loratadine, cetirizine, azelastine, and fexofenadine are not associated with torsades de pointes or other ventricular arrhythmias.[119]

Concomitant with the H_1R antagonists reclassification, two neutral H_1R antagonists have been identified as well (see also Chapter 3, page 47).[92] Histabudifen (pK_i=5.8) and histapendifen (pK_i=5.9) (Figure 5C), being histaprodifen-like compounds, exhibit hybrid structures composed of the endogenous agonist histamine combined with the classical H_1R antagonist pharmacophore. Thus, the strategy of modifying the agonists structures, already successfully used to identify neutral H_2 and H_3 receptors antagonists (see sections 1.2.3.3 and 1.2.4.3), has proven to be effective for obtaining neutral histamine receptor antagonists as well.
Additionally, lysergol (pK_i=5.6, Figure 5C), an analogue of lisuride, seems to lack intrinsic H_1R activity,[84] and thus represents a potential lead structure for a new class of neutral H_1R antagonists.
All the newly identified neutral H_1R antagonists, despite their moderate H_1R affinity, might be useful pharmacological tools and may as well serve as a starting point for the development of high-affinity and potent neutral H_1R antagonists, the availability of which will allow the assessment of the importance of constitutive H_1R activity *in vivo* as well as the requirements of inverse H_1R agonistic properties for the therapeutic value of H_1-antihistamines.

1.2.3 Histamine H_2 Receptor

1.2.3.1 Distribution, function and signal transduction mechanisms

H_2Rs have been detected in a variety of tissues including brain,[80,120] gastric cells[121,122] and cardiac tissue.[123,124] H_2Rs have a potent stimulatory effect on gastric acid secretion, and the inhibition of this process by H_2R antagonists has provided evidence for an important physiological role of histamine in the regulation of gastric acid secretion.[54] High concentrations of histamine are also present in cardiac tissues of most animal species and can mediate positive chronotropic and inotropic effects on atrial or ventricular tissues via H_2R stimulation.[54,125,126] H_2R-mediated smooth muscle relaxation has also been documented in airway, uterine and vascular smooth muscle.[54,127] Finally, H_2Rs can influence a variety of

functions within the immune system.[64] In the CNS, H_2R activation can inhibit nerve cells and block the long-lasting after-hyperpolarization and the accommodation of firing in rodents[128] and human brain.[129]

The H_2R gene was first cloned by Gantz and colleagues from canin gastric parietal cDNA,[49] and soon after that in rat,[130] human,[131] guinea pig[132] and mouse.[133]

It is generally accepted that H_2Rs couple to adenylyl cyclase via the GTP-binding protein G_s,[64,75,134] and H_2R-mediated cAMP accumulation has been observed in many cell types.[134] There are also reports of H_2Rs coupling to other signalling systems, such as PLC and intracellular calcium signalling via the activation of $G\alpha_q$ proteins in certain cell systems.[135-137]

1.2.3.2 Constitutive H_2R signalling and reclassification of H_2R antagonists

The H_2R was the first member of the histamine receptor family for which constitutive activity was demonstrated, in fact expression of the H_2R in CHO cells, already at moderate densities, results in agonist-independent elevation of cAMP levels, thus leading to spontaneous H_2R activity.[21,29]

As already seen with H_1R antagonists (see section 1.2.2.3), constitutive H_2R activity caused a reclassification of H_2R antagonists. Cimetidine and ranitidine (Figure 6B), previously thought to act as competitive H_2R antagonists, actually function as inverse agonists.[21,29] The inverse agonism displayed appears to be a mechanistic basis for the observed H_2R-antagonist-induced H_2R upregulation in CHO cells. As already discussed in section 1.1.3, these findings altered the view regarding the action of H_2 antagonists and have been proposed to have implication for effective drug therapy.[21] Despite the widespread therapeutic use of H_2R antagonists in humans, no data on the (patho)physiological significance of constitutive H_2R activity and/or H_2R upregulation following chronic inverse H_2R agonists treatment, are currently available.

1.2.3.3 H_2R specific ligands

5-Methylhistamine (Figure 6A) was the first agonist described that had a certain selectivity for the H_2R,[54] although more potent and selective H_2R agonist are now available, e.g. impromidine (Figure 6A),[138] sopromidine[139] and arpromidine (Figure 6A).[140] The latter one and analogues are potential candidates for treatment of congestive heart failure. Another potent H_2R agonist, amthamine (Figure 6A), has been derived as an analogue of dimaprit (Figure 6A)[141] by considering cyclic forms of the isothiourea group.[142]

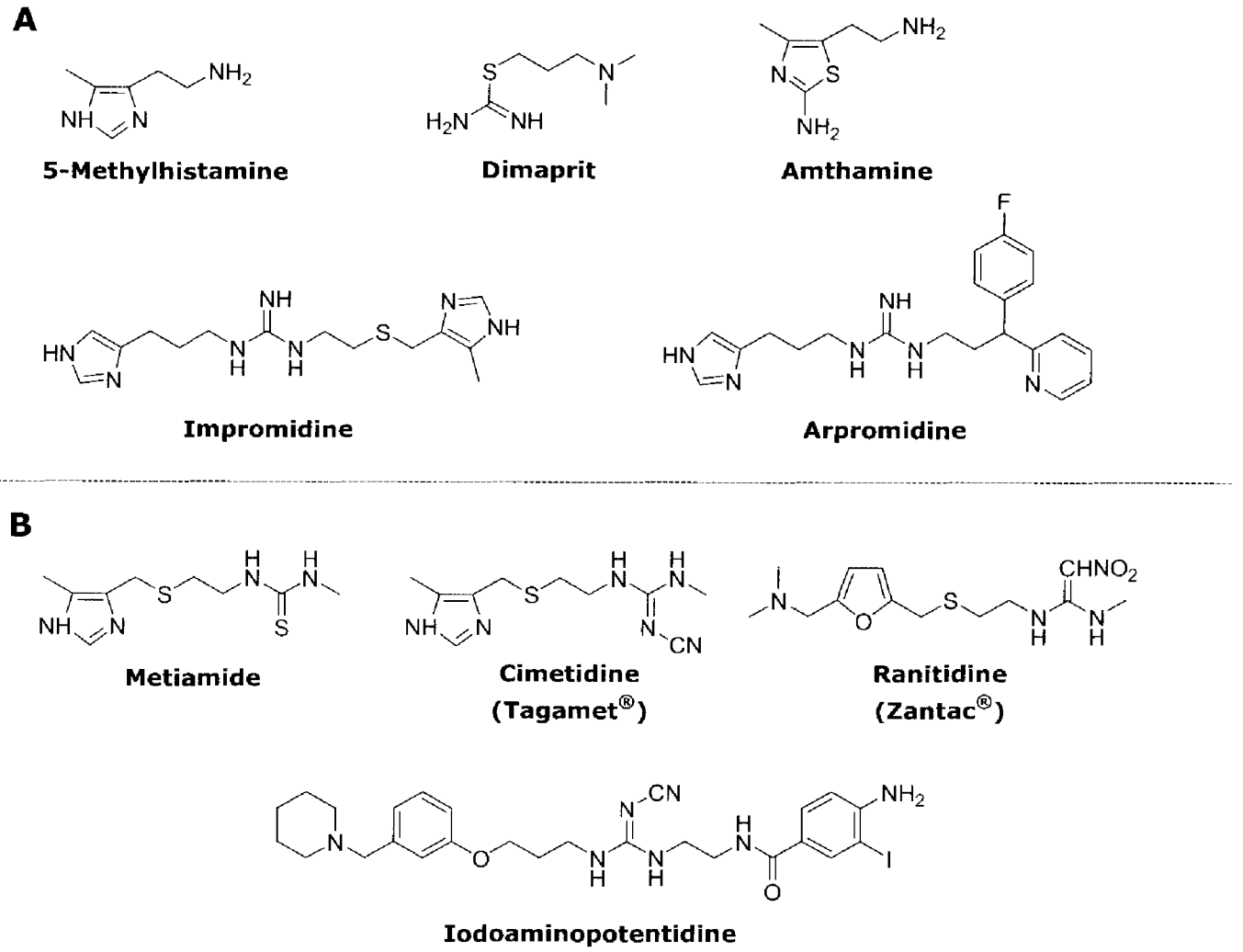


Figure 6. Structures of several H_2R ligands. A, H_2R agonists. B, Inverse H_2R agonists. C, Neutral H_2R antagonists.

The initial definition of the H_1- and H_2-subclasses of histamine receptors by Ash and Schild[53] and Black and colleagues[54] led to a successful search for H_2R selective antagonists with clinical relevance for the treatment of peptic ulcers. Burimamide (Figure 6C) was the first compound developed that showed H_2R selectivity,[54] but was later proved to be actually more potent as an H_3R antagonist.[55] Cimetidine[143] and metiamide[144] (Figure 6B) were developed directly from burimamide, and since then a large number of compounds with H_2R antagonistic properties have been developed, among which ranitidine[145] (Figure 6B) and tiotidine.[146] These compounds have been extensively used for characterization purposes. Iodoaminopotentidine (Figure 6B) is

one of the most potent H_2R antagonists available and has been employed as a successful radioligand.[147]

Just as for H_1R antagonists, recognition of constitutive H_2R activity resulted in the reclassification of cimetidine, ranitidine (Figure 6B), tiotidine and famotidine as inverse H_2R agonists.[21,29] On the other hand, burimamide (Figure 6C) behaved as a neutral H_2R antagonist on the rat H_2R[21] or as a weak partial agonist on the human H_2R,[29] both transfected in CHO cells. Remarkably, whilst burimamide, being a neutral H_2R antagonist, did not induce upregulation of the rat H_2R after long-term exposure,[21] it did induce, like inverse H_2R agonists, an upregulation of the human H_2R following prolonged treatment, which suggested a structural instability of the constitutively active human H_2R in transfected CHO cells. Occupation of the H_2R by any ligand (thus also the weak partial agonist burimamide) reduced the instability, resulting in higher cellular expression level.[29]

1.2.4 Histamine H_3 Receptor

1.2.4.1 Distribution, function and signal transduction mechanisms

The third histamine receptor was discovered in 1983 by Arrang and coworkers based on classical pharmacological experiments.[55] This receptor subtype confirmed the function of histamine as a neurotransmitter. The H_3R mediates the inhibition of synthesis and release of histamine from histaminergic neurons via a negative feedback loop[148,149] but also exerts modulatory effects on other neurotransmitter systems, e.g. the cholinergic,[150,151] dopaminergic,[152] noradrenergic[153] and serotoninergic,[154] glutamatergic[155] and peptidergic[156] systems, in both the central and peripheral nervous systems.

Despite the cloning of the genes encoding the H_1 and H_2 receptors in 1991,[48,49] it lasted until 1999 before Lovenberg and co-workers succeeded in cloning the H_3R gene,[50] which revealed very low homology with other GPCRs. Overall homology with the H_1R and H_2R is only 22% and 21.4%, although a greater degree of homology can be observed within TMs (27% for H_1R and 31% for H_2R). The H_3R is expressed in peripheral tissues and predominantly in the CNS, with greatest expression in the thalamus and caudate nucleus.[50] Molecular characterization of H_3Rs in other species has been extensively investigated and the rat,[157] guinea pig,[158] monkey[159] and mouse[160] H_3R genes have been identified as well.

Isoforms with different binding, signalling properties and brain expression levels have been identified in humans, rats, guinea pigs and mice,[157,158,161-164] but not in monkeys.[159] The existence of multiple splice isoforms of the H_3R suggests different functions of these isoforms.

It has also been recently shown that H_3Rs from different species exhibit different pharmacological profiles. Although the human and rat H_3Rs are highly conserved, exhibiting

93% identity of their amino acid sequences,[50,157,161,165] distinct ligand binding properties have been observed.[157,166-168]

In vitro, the H_3R is negatively coupled to cAMP via adenylyl cyclase (AC) by members of the G_i family of G proteins, such as $G\alpha_i$ and $G\alpha_o$.[169] In addition, a variety of other effector pathways can be activated by the H_3R, including the mitogen-activated protein kinase (MAPK) pathway[161] and the Na^+/H^+ exchanger.[170]

1.2.4.2 Constitutive H_3R signalling

The recombinant human and rat H_3Rs expressed at physiological densities have been shown to display a high degree of constitutive activity.[26,171,172] Consistent with the physiological relevance of the process, *in vivo* evidence of constitutive receptor activity (that was not due to overexpression of receptors) has been provided for the H_3R in rodent brain.[26] Suppression of this activation has been described as well, thus allowing the characterization of many H_3R antagonists more accurately as inverse agonists; in fact, inverse agonists acting at the H_3 (auto)receptor enhanced K^+-induced release of histamine in rat brain synaptosomes.[26] In a clinical context, these results have led to the suggestion that inverse agonists might be preferred to neutral antagonists as "cognitive enhancers".[173]

1.2.4.3 H_3R specific ligands

The H_3R has been recognized as a potential therapeutic target.[57,174,175] H_3R antagonists have been proposed to be potential drugs for the treatment of several diseases and CNS disorders, such as attention-deficit hyperactivity disorder (ADHD),[176,177] Alzheimer's disease,[178] epilepsy,[179-181] schizophrenia[181,182] and obesity,[183-185] whereas the therapeutic potential of H_3R agonists has been shown for myocardial ischemia,[186] inflammatory[187] and gastric acid related diseases,[188] migraines and sleep disorders.[189]

A wide array of potent and selective H_3R ligands have been described and used as pharmacological tools. However, no H_3R ligands have reached clinical practice yet.

At the H_3R, histamine itself is a highly active agonist. Mono- or dimethylation of the terminal amino function results in compounds that are more active and H_3R selective with regard to H_1 and H_2 receptors, than histamine. Methylation of the α-carbon atom of the ethylamine side chain drastically increases the H_3R potency, which however resides only in the R-isomer, the corresponding S-isomer being approximately 100-fold less potent. Since the methylation leads to highly reduced activity at both the H_1 and H_2 receptors, R-(α)-methylhistamine (RAMH, Figure 7A) is a very selective H_3R agonist,[166] even though it has recently been reported to interact with the H_4R as well.[59] Nonetheless, in combination with its less active S-isomer, this

compound has proven to be a highly useful tool for the pharmacological characterisation of H_3R-mediated effects.[190] Tritiated derivatives of N^{α}-methylhistamine and RAMH are currently available as radiolabelled H_3R agonists.[166,191] For potent H_3R agonism, the amine function of histamine can either be replaced by an isothiourea group, as in imetit (Figure 7A),[192,193] or incorporated in ring structures to produce compounds such as immepip (Figure 7A).[194] RAMH, imetit and immepip are very active both *in vitro* and *in vivo*. Moreover, whilst RAMH shows some H_1R and α_2-adrenoceptor agonistic activity, and imetit acts as a 5-HT_3 agonist, immepip is devoid of those activities.[195-197] Recently, immethridine (Figure 7A), a histamine analogue in which the amine function of the side chain has been incorporated in a pyridine ring, and VUF 5826 (Figure 7A), a N^{α},N^{α}-dimethylated higher homologue of histamine (see also Chapter 4, page 79), have been identified as potent and selective H_3R agonist, displaying respectively a 300-fold and 250-fold selectivity over the H_4R.[198,199] Methimepip (Figure 7A), an immepip derivative methylated on the side chain nitrogen, exhibits an even greater H_3R/H_4R specificity ratio.[200] These compounds may become useful pharmacological tools for investigating the H_3R and may as well serve in modelling and mutational studies.
In the line of H_3R agonists development, other recent series of compounds lacking the basic element in the side chain, such as FUB 407 (Figure 7A),[201] should also be mentioned. These compounds display partial agonism *in vitro*, depending on the test system, and partial to full agonism *in vivo*.[202-204]

Several potent H_3R antagonists have been described as well.[101,102,205] However, many H_3R ligands previously described as antagonists have now been recharacterized as inverse agonists, sometimes with different intrinsic activities. Thioperamide[166] and clobenpropit (Figure 7B),[193] formerly classified as H_3R antagonists, are in fact very potent H_3R inverse agonist and are often considered to be the standard H_3R antagonists, despite their recently identified affinity for the H_4R as well. Iodophenpropit (Figure 7B), an iodinated analogue of clobenpropit, is also a potent H_3R inverse agonist and a valuable tool in pharmacological studies. Moreover, its radiolabelled ^{125}I derivative, can be used as a highly sensitive H_3R radioligand.[206]
Ethers such as proxyfan (Figure 7C, see also below)[207] or cyproxyfan (Figure 7C),[208] are well known and useful pharmacological tools for the study of the H_3R-mediated effects, while the chiral alkyne derivative GT-2331[209] (cipralisant, Perceptin®, Figure 7B), initially reported to be an antagonist[210] and recently reassessed as partial H_3R agonist,[211] is currently under clinical evaluation and might yield the first drug targeting the H_3R.

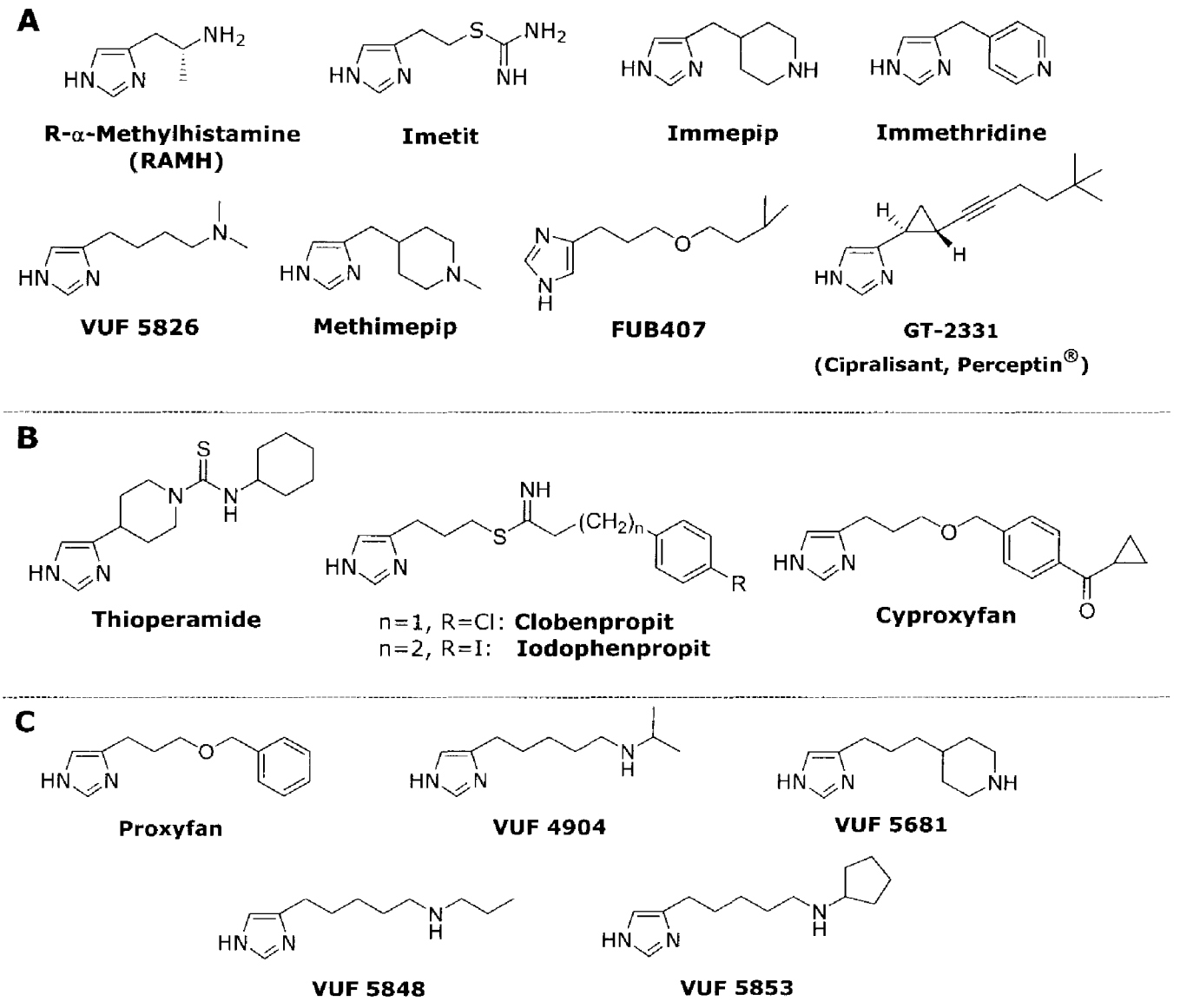


Figure 7. Structures of several H_3R ligands. A, H_3R agonists. B, Inverse H_3R agonists. C, Neutral H_3R antagonists.

Some neutral H_3R antagonists have so far been described. Proxyfan (Figure 7C), a high affinity H_3R ligand, opposing the actions of both agonists and inverse agonists, and lacking a pharmacological effect itself, was actually the first compound to be identified as a neutral antagonist for the H_3R.[26] However, based on its paradoxical effects in different assays, proxyfan has been recently identified as a so-called protean H_3R ligand, with the ability to act as agonist, neutral antagonist or inverse agonist at recombinant H_3Rs expressed in the same CHO cells. In support of the physiological relevance of the process, proxyfan has also been shown to behave as a protean agonist at native H_3Rs known to display constitutive activity.[212] On neurochemical and behavioural responses in rodents and cats, proxyfan displays a spectrum of activity ranging from full agonism to full inverse agonism, making it a powerful tool to investigate active receptor conformations not only in cells but also in tissues. The

therapeutic interest of protean agonists is hard to predict. They might be helpful to maintain a constant level of stimulation of the receptor, but their effect may vary among systems and patients and/or lead to paradoxical responses.

VUF 4904 and VUF 5681 (Figure 7C) have also been recently reported to behave as neutral H_3R antagonists at the human H_3R expressed in SK-N-MC cells.[171,213] Moreover, several neutral H_3R antagonists have been identified within a series of higher homologues of histamine with different substitutions on the terminal nitrogen atom (see also Chapter 3, page 47)[199]. The recognized neutral antagonists displayed variable H_3R affinities and H_3R/H_4R specificity ratios, but two of them, namely N^{α}-ethylimpentamine (VUF 5848) and N^{α}-cyclopentylimpentamine (VUF 5853) (Figure 7C), showed nanomolar H_3R affinity. In view of the many potential therapeutic applications of H_3R antagonists,[57] the availability of high affinity neutral H_3R antagonists such as VUF 5848 and VUF 5853 may help in establishing whether inverse agonists or neutral antagonists will be favored for clinical applications.

1.2.5 Histamine H_4 Receptor

1.2.5.1 Distribution, function and signal transduction mechanisms

Soon after the cloning of the H_3R gene, a fourth histamine receptor gene was cloned by different groups simultaneously.[51,59-63] The H_4R shows 37% to 43% overall homology to H_3Rs depending on species, which rise to 58% identity in transmembrane regions. With the information on the human H_4R cDNA, the mouse, rat, guinea pig[214] and pig[215] H_4R cDNAs have been identified. H_4Rs from different species share 65-72% homology with the human H_4R and exhibit substantial pharmacological species variation either in binding affinity or signal tranduction responses.[214,215]

Notably, the distribution of the H_4R is quite different from that of the H_3R. In contrast to a nearly exclusive brain localization for the H_3R, the H_4R shows highest levels in bone marrow and leukocytes, particularly eosinophils and neutrophils, with moderate levels in spleen and small intestine. Mast cells have also been reported to contain the H_4R.[62] Preliminary expression studies reported the absence of the H_4R in the CNS,[51,61,63] although is situ hybridisation studies in mouse[62] and Rnase protection assays in human samples[59] yielded evidence for a brain localization. The tissue expression profile of the H_4R combined with its interaction with cytokines (e.g. TNF-α) and various interleukins,[216] suggests an important role in inflammatory processes, regulation of immune function (particularly with respect to allergy and asthma) and hematopoiesis.

Like the H_3R, the H_4R seems to couple to pertussin toxin sensitive $G_{i/o}$, thereby inhibiting forskolin-activated cAMP formation,[51,59,62] even though other effector pathways have also been proposed, including the activation of MAPKs[61] and ligand-induced calcium mobilization.[51]

1.2.5.2 Constitutive H_4R signalling

The H_4R has been suggested to exhibit a significant level of constitutive activity, indicated by the elevated basal level of both [^{35}S]GTPγS binding and MAPK phosphorylation seen in cells expressing the human H_4R as compared with wild-type cells.[61] It is unlikely that the observed constitutive H_4R activity is due to over-expression of this receptor, as similar results were obtained from both transient transfections and from a stable cell line expressing relatively low levels of receptor. It remains to be determined if the H_4R, in its native cellular environment, exhibits a similar degree of constitutive activity, and if so, what is the functional significance of such activity.

1.2.5.3 H_4R specific ligands

Many compounds described as ligands for H_3Rs display activity also for H_4Rs. Given the structural similarities of the two receptors, it is not surprising that their pharmacologies overlap. The high-affinity H_3R agonists also have H_4R agonist activity, but with a reduced potency. This seems to be true for many of the imidazole-containing compounds, although it has been tested only for a limited number of compounds. Most notable, the potency of the agonists histamine and RAMH (Figure 7A) is reversed at the H_4R in comparison with the H_3R, with histamine being more potent than RAMH at the H_4R, whereas RAMH is more potent at the H_3R.[59,61]

Thioperamide (Figure 7B), the prototypical H_3R inverse agonist, displays also an appreciable H_4R antagonistic activity,[61] though with 5- to 10-fold lower potency. Some data even suggest that this compound may be an inverse agonist at H_4Rs,[59] similar to results showing this effect on H_3Rs.[26] Besides thioperamide, only one other ligand, VUF 4742 (Figure 8B) has so far been reported to exhibit inverse H_4R agonism.[217]

The inverse H_3R agonists clobenpropit and burimamide (Figure 7B) also have a lower affinity for the H_4R, and moreover show partial agonistic activity at the H_4R.[59,61] Impromidine, dimaprit (Figure 7A) and the antipsychotic drug clozapine (Figure 8A) shows higher (though still moderate) affinity at the H_4R than at the H_3R.[51,59] Interestingly, two of the compounds, impromidine and dimaprit, are better known for their H_2R agonistic effects. Impromidine and dimaprit behave as partial agonists at the H_4R, whereas clozapine appears to be a full agonist.[59]

Only a few compounds showing a higher H_4R specificity over the closely related H_3R have been described to date. Those include the imifuramine derivative OUP-16 (Figure 8A), a full H_4R agonist with 41-fold higher potency at the H_4R than at the H_3R,[218] the non-imidazole H_4R antagonist JNJ 7777120 (Figure 8C),[219] which also exhibits anti-inflammatory activity *in vivo*,[220] and its benzimidazole analogue VUF 6002 (Figure 8C).[221]

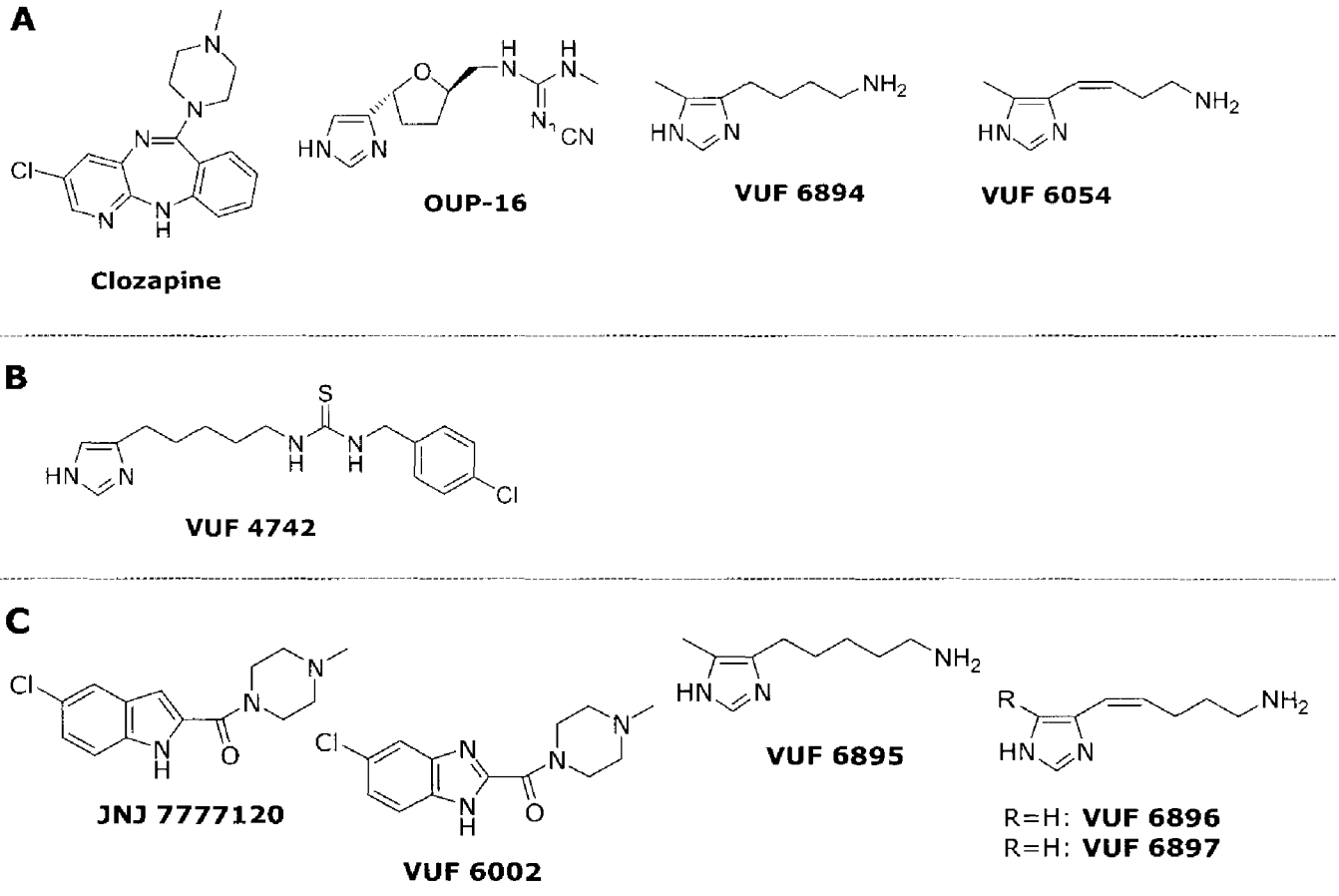


Figure 8. Structures of several H_4R ligands. A, H_4R agonists. B, Inverse H_4R agonists. C, Neutral H_4R antagonists.

In the quest for novel ligands selectively addressing the H_4R over the H_3R we recently prepared a series of histamine derivatives bearing a longer and/or unsaturated side chain as well as the corresponding 5-methylated derivatives. In this series we identified 5-methylhistamine (Figure 6A) and its analogues VUF 6894 and VUF 6054 (Figure 8A) as compounds specifically targeting the H_4R over the H_3R[222] (see also Chapter 5, page 115). Moreover, compounds VUF 6895, VUF 6896 and VUF 6897 (Figure 8C) were recognized as neutral antagonists at the human H_4R transfected in SK-N-MC cells.[222] This series of compounds possess interesting structure-activity relationships and could thus become valuable pharmacological tools as well as serve for modelling and mutational studies.

Interestingly, we also recently discovered that the non-imidazole compound JNJ 777120 (Figure 8C), reported as an H_4R antagonist,[219] actually behaves as a neutral H_4R antagonist in SK-N-MC cells transfected with the human H_4R.[221] Similarly, we identified its benzimidazole analogue (VUF 6002, Figure 8C)[221] and the H_3R inverse agonist iodophenpropit (Figure 7B)[223] as neutral H_4R antagonists as well.

1.3 Fluorescent ligands for the study of receptors

The GPCR family of receptors represents a very dynamic set of biologically active proteins. Traditional beliefs are being overturned as new information regarding the signalling properties, cycling, fate and heterologous interactions between different classes of receptors are acquired. It is now clear that GPCRs can exist in either diffuse or clustered distributions. Receptors are not confined to the cell surface and are sequestered and internalised in an agonist-dependent fashion. Constitutively active receptors may trigger internalisation without the presence of an agonist. Finally, receptors may be internalised passively due to constitutive endocytosis. These receptors, which play key physiological roles, are major target for drugs used in the treatment of a huge number of diseases. There is therefore an urgent need to be able to visualize and quantify the binding of drug molecules to their target receptors in individual living cells.

Fluorescent techniques are useful methods to study the location or concentration of molecules as they are non-invasive, can be performed with high sensitivity and can offer good specificity; in addition, they offer a way to dodge several of the inconveniences associated with radioligand binding assay, such as high costs, potential health hazards, waste disposal problems, and the related need for large number of cells with relatively high number of receptor expression.[224]

Fluorescence from living cells can be detected with spectroscopic[225] or microscopic techniques. A specific microscopy approach, already proven very useful in the visualization of fluorescent ligand-receptor complexes,[226-228] is confocal laser-scanning microscopy. In conventional microscopy, fluorescent images are degraded by light emitted or scattered by tissue that is out of the plane of focus. This produces an image of decreased resolution and reduced contrast. Confocal imaging, based on a principle developed by Minsky,[229] requires illumination and detection systems to be focused on the same small region within the tissue. Light from the illuminated volume is sampled so that the fluorescent signals arise from this area of tissue. Fluorescent signals that arise from outside the sampled area are "rejected" by a spatial filter, such as a pinhole. As both the illumination and detection systems are on the same focal plane, they are said to be confocal.[230,231]

Fluorescent tagging of pharmacological agents has proven to be a useful tool for the investigation of the interactions of different receptors with their ligands in complement to

classical methods such as radioligand binding and site-directed mutagenesis.[224,232,233] Fluorescent ligands have been used since the mid-1970s and offer several advantages over traditional radioligand binding techniques.[224] Indeed, the use of fluorescent ligands can provide a multiplicity of informations such as the mechanism of ligand binding,[234,235] the localization, movement and internalisation of receptors in living cells,[236,237] the distances between ligands and fluorescently labeled amino acids,[238,239] and the physical nature of the binding pocket.[240,241] Moreover, fluorescent ligands have the advantage of allowing direct visualization of the receptor using microscopy and hence the actual location of receptors within single living cells can be followed over time. Such an approach has been used successfully for a number of GPCRs.[224,242,243] For example, BODIPY-TMR-CGP has been recently reported as a long-acting fluorescent β_2-adrenoceptor partial agonist that can be used to label β_2-adrenoceptor in the plasma membrane of living mammalian cells.[226] A fluorescent quinazoline derivative (QABP) acting as a competitive antagonist at α_1-adrenoceptor was used in living cells to localize receptors and to quantify receptor binding characteristic.[244] The first fluorescent antagonist for human 5-HT_4 receptors has also been lately synthesised and effectively used in fluorescence microscopy experiments to label h5-$HT_{4(e)}$ receptor isoform transfected in C6 glial cells.[245]
Furthermore, agonists that retain their agonist efficacy after tagging with a fluorophore offer an opportunity to study the dynamics of the receptor activation process.[246]
Briddon *et al.* recently reported about the use of a fluorescent antagonist as well as a fluorescent agonist at the adenosine A_1 receptor, for the quantitative analysis of the formation and diffusion of the ligand-receptor complex in single living cells using fluorescence correlation spectroscopy (FCS).[227,228] Prior to these studies, FCS had been used to measure ligand binding to cell surface receptor on both the tyrosine kinase and GPCR superfamilies.[247-250] However, those studies were performed with peptide ligands, which bind to the extracellular region of the receptor. No similar studies with ligands binding to the intramembrane region of the GPCRs had been performed until this report, underscoring the potential of fluorescent ligands for the study of receptors.
Another fine example of what can be achieved through the use of confocal microscopy and multiple fluorophore-tagged ligands has been provided by Pick and colleagues,[251] who used compounds from a recently developed family of fluorescent antagonists (based on GR119566) for the 5-HT_3 receptor, a multimeric ligand-gated ion channel, for time course studies on receptor cluster formation. This approach, using spectrally appropriate ligands, should be useful for the study of GPCRs as well.
Fluorescent ligands have also been used to study dimerization or oligomerisation of GPCRs by fluorescence resonance energy transfer (FRET).[249,252,253] Oligomerization or dimerization of GPCRs has emerged has an important theme in signal transduction, a concept that has recently gained widespread interest due to the application of direct and non-invasive biophysical techniques such as FRET. This imaging technique is a distance-dependent

interaction between two molecules in which the excitation energy of one molecule (the donor) can be transferred to the other without emission of light energy from the donor molecule. For FRET to occur molecules must be in close proximity (no greater than 100 Å), and the absorption spectrum of the recipient molecule must overlap the emission spectrum of the donor. FRET studies have indeed shown unequivocally that several types of GPCR can form dimers or oligomers in living cells.[249,252,253]

Thus, the combination of sophisticated imaging technologies and the diverse array of fluorescent probes makes fluorescence microscopy the tool with which to assess not simply the presence or location of molecules within cells and tissues, but also many of the dynamic features of cell function, such as receptor-mediated mechanisms in living tissues. An important and present key issue is how a gene product that is artificially introduced into a tissue will behave in relation to its native counterpart, thus the next stage in the evolution of these technologies looks likely to be an advance towards *in vivo* measurements. Important steps towards this end have already begun to take place.[254]

1.3.1 Fluorescent ligands for the histamine receptors

Pioneer work on the synthesis of fluorescent histamine ligands and their detection in live cells has been done in 1986 by Petty and Francis, who synthesised and studied the fluorescent conjugate histamine-fluorescein.[255] Clusters of mobile receptors on the cell surface of polymorphonuclear leukocytes were detected. Furthermore, fluorescein-histamine binding was inhibited in the presence of histamine and cimetidine, confirming that conjugation of the fluorophore does not markedly alter its selectivity. In this study it was concluded that the development of H_1R and H_2R specific fluorescent labels should allow simultaneous localization of these distinct receptor.

However, the synthesis of H_1R and H_2R specific fluorescent ligands has been achieved only recently, with the work of Li and colleagues.[256,257] Fluorescent H_1R ligands were obtained by conjugating normepyramine (a demethylated analogue of mepyramine) with various fluorophores by different functional groups and through spacer of various lengths.[256] The general structure of the synthesised compounds is depicted in Figure 9A. The introduced fluorophore moieties included 5-carboxyfluorescein (5-FAM), 6-carboxyfluorescein (6-FAM), BODIPY® 493/503, 5-carboxytetramethylrhodamine (5-TAMRA), Rhodamine Green™ carboxylic acid (5(6)-CR 110), 5-carboxynaphtofluorescein, 4-chloro-7-nitrobenzofurazan (NBD), NBD-X and dansyl. The fluorescent compounds were investigated for H_1R antagonistic activity and it was concluded that rather potent fluorescent-labelled H_1R antagonists could be obtained by connecting normepyramine and fluorescein or NBD via a spacer of appropriate length. Bulky fluorophores are tolerated when linked to the affinity-conferring partial structure by an

appropriate spacer. It is thus conceivable that the various bulky dyes do not interact with amino acids of the transmembrane H_1R domains, but are extending in the extracellular space.

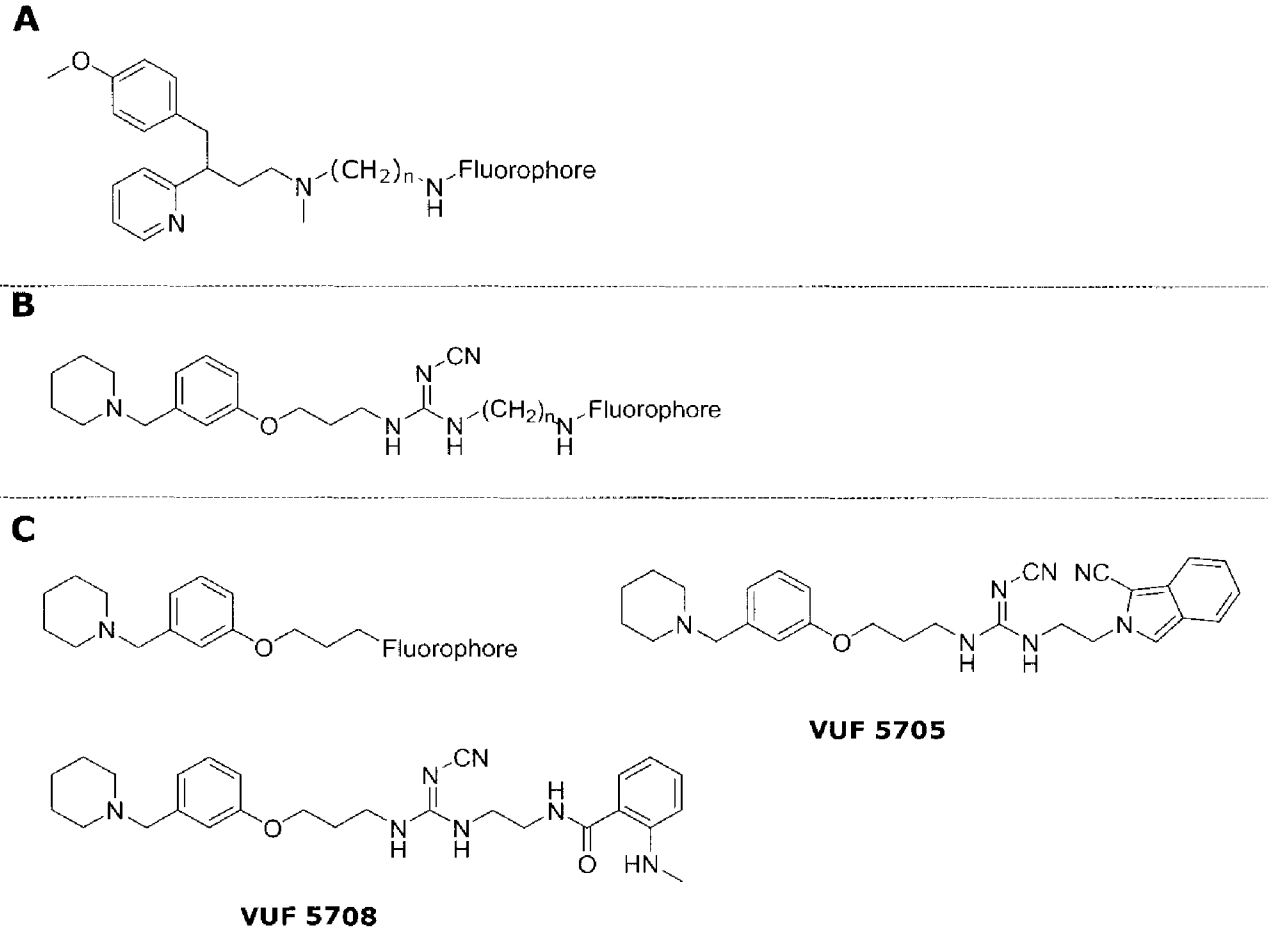


Figure 9. Fluorescent ligands for the histamine receptors. A, Fluorescent H_1R ligands: general structure of the fluorescently-labelled mepyramine analogues. B, Fluorescent H_2R ligands: general structure of the fluorescently-labelled aminoalkyl-substituted *N*-[3-(3-piperidin-1-ylmethylphenoxy)propyl]cyanoguanidine derivatives. C, Fluorescent H_2R ligands: general structure of the fluorescently-labelled 3-[3-(piperidinomethyl)phenoxy]propyl derivatives and structures of the fluorescent *N*-[3-(3-piperidin-1-ylmethylphenoxy)ethyl]cyanoguanidine derivatives VUF 5705 and VUF 5708.

Fluorescently labelled H_2R antagonists were designed by analogy with the approach that had already been successfully applied to the development of the high affinity H_2R radioligand [^{125}I]iodoaminopotentidine (Figure 6B).[147] As spacefilling residues such as radiolabelled partial structures including spacer groups are tolerated in the aminopotentidine series, the title compounds were structurally derived from aminoalkyl-substituted *N*-[3-(3-piperidin-1-ylmethylphenoxy)propyl]cyanoguanidine.[257] The general structure of the synthesised compounds is depicted in Figure 9B. In this case the fluorescent moieties employed to derivatise the partial structure were 5-carboxyfluorescein (5-FAM), 6-carboxyfluorescein (6-FAM), acridine-9-carboxylic acid (9-AC), NBD, NBD-X and dansyl. The fluorescent conjugates

were investigated for H_2R antagonistic activity, pointing out that potent fluorescent-labelled H_2R antagonist of the *N*-[3-(3-piperidin-1-ylmethylphenoxy)propyl]cyanoguanidine type could be obtained by derivatisation of corresponding primary amines having appropriate spacer lengths (6-8 methylene groups) with NBD dyes. All the other fluorophores were inappropriate to confer high H_2R antagonistic activity, in contrast with the series of mepyramine-related H_1R antagonists (see above), where both the NBD and carboxyfluorescein moiety were found to have an activity-enhancing effect. As the NBD group was the smallest among the fluorescent labelling agents tested in this study, it was speculated that the bulk of the fluorophore and not the length of the connecting chains is the limiting factor in the case of H_2R antagonists related to iodoaminopotentidine.

These studies suggest that it may be possible to combine fluorescent-based methods for functional studies (e.g. calcium assay) and for determination of binding constants of GPCR ligands. The combination of both may be potentially useful in high-throughput screening to get informations on affinities and agonistic/antagonistic properties of potential drug candidates in one assay. Moreover, fluorescent probes such as the fluorescein-labelled mepyramine analogues may be useful to study the GPCRs in tissue preparations and in cells.

Fluorescent ligands for the H_2R have also been synthesised derivatising partial structure from the aminopotentidine series[147] with fluorophoric groups.[258] In particular, 3-[3-(piperidinomethyl)phenoxy]propyl and *N*-[3-(3-piperidin-1-ylmethylphenoxy)alkyl]cyano guanidine were linked to several fluorophoric groups (see general structures in Figure 9B,C), such as dansyl, NBD, 1-cyanoisoindole, N-methylanthranilate, 1-cyano-indolizine-2-carboxylate, ethyl indolizine-2-carboxylate-1-carboxylate and BODIPY® FL. The prepared conjugates were assayed for their H_2R binding properties, which resulted in pK_i values ranging from 5.99 to 8.85. The *N*-[3-(3-piperidin-1-ylmethylphenoxy)alkyl]cyanoguanidine partial structure gave in general fluorescent derivatives displaying a higher affinity compared to the fluorescent conjugates prepared with the 3-[3-(piperidinomethyl)phenoxy]proyl partial structure. The optimum length of the alkyl chain connecting the cyanoguanidine moiety to the fluorophore seemed to be of two methylene groups. The highest H_2R affinities resided in *N*-[3-(3-piperidin-1-ylmethylphenoxy)ethyl]cyanoguanidine bearing either a cyanoindolizine fluorophore (VUF 5705, Figure 9C) or a methylanthranilate group (VUF 5708, Figure 9C). Thus, it was demonstrated that it is possible to obtain high affinity fluorescent H_2R ligands, which should be useful pharmacological tools to investigate ligand receptor interactions in binding assays and functional studies.

To date, no fluorescently labelled H_3R and H_4R ligands have been reported in the literature.

The green fluorescent probe BODIPY® FL-histamine[259] is synthesised and sold by Molecular Probes. We have attempted to label human H_3Rs in transfected CHO cells with this ligand (unpublished results, see Chapter 7, page 163), but unfortunately did not succeed in this

purpose, owing to the rapid internalisation of the ligand in the cytoplasm of the cells, possibly caused by the inadequate physico-chemical properties of the conjugate (i.e. high lipophylicity). However, fluorescent conjugates specifically targeting the H_3R and H_4R should represent valuable tools for the visualization and the study of binding and activation mechanisms of these recently cloned receptors in different isoforms, cell types and tissues. Thus, synthetic pathway and fluorescent conjugates for the achievement of suitable fluorescent H_3R and H_4R probes will have to be investigated in the future.

References

(1) Gether, U. Uncovering Molecular Mechanisms Involved in Activation of G-Protein Coupled Receptors. *Endocr. Rev.* **2000**, *21*, 90-113.

(2) Pierce, K. L.; Premont, R. T.; Lefkowitz, R. J. Seven-transmembrane Receptors. *Nat. Rev. Mol. Cell. Biol.* **2002**, *3*, 639-650.

(3) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science* **2000**, *289*, 739-745.

(4) Costa, T.; Hertz, A. Antagonists with Negative Intrinsic Activity at δ-Opioid Receptors Coupled to GTP-binding Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7321-7325.

(5) Milligan, G.; Bond, R. A.; Lee, M. Inverse Agonism: Pharmacological Curiosity or Potential Therapeutic Strategy? *Trends Pharmacol. Sci.* **1995**, *16*, 10-13.

(6) Leurs, R.; Smit, M. J.; Alewijnse, A. E.; Timmerman, H. Agonist-independent Regulation of Constitutively Active G-protein Coupled Receptors. *Trends Biochem. Sci.* **1998**, *23*, 418-422.

(7) Milligan, G. Constitutive Activity and Inverse Agonists of G Protein-Coupled Receptors: a Current Perspective. *Mol. Pharmacol.* **2003**, *64*, 1271-1276.

(8) Bailey Patent Status of the Therapeutically Important G-protein Coupled Receptors. *Expert Opin. Ther. Patents* **2001**, *11*, 1861-1887.

(9) Samama, P.; Cotecchia, S.; Costa, T.; Lefkowitz, R. J. A Mutation-induced Activated State of the β2-Adrenergic Receptor. Extending the Ternary Complex Model. *J. Biol. Chem.* **1993**, *268*, 4625-4636.

(10) Leff, P. The Two-state Model of Receptor Activation. *Trends Pharmacol. Sci.* **1995**, *16*, 89-97.

(11) Kenakin, T. P. The Classification of Seven Transmembrane Receptors in Recombinant Expression Systems. *Pharmacol. Rev.* **1996**, *48*, 413-416.

(12) Ehlert, F. J.; Roeske, W. R.; Gee, K. W.; Yamamura, H. I. An Allosteric Model for Benzodiazepine Receptor Function. *Biochem. Pharmacol.* **1983**, *32*, 2375-2383.

(13) Kenakin, T. P. Inverse, Protean and Ligand-selective Agonism: Matters of Receptor Conformation. *FASEB J.* **2001**, *15*, 598-611.

(14) Milligan, G.; Bond, R. A. Inverse Agonism and the Regulation of Receptor Number. *Trends Pharmacol. Sci.* **1997**, *18*, 468-474.

(15) Daeffler, L.; Landry, Y. Inverse Agonism at Heptahelical Receptors: Concept, Experimental Approach and Therapeutical Potential. *Fundam. Clin. Pharmacol.* **2000**, *14*, 73-87.

(16) De Ligt, R. A. F.; Kourounakis, A.; Ijzerman, A. P. Inverse Agonism at G-protein Coupled Receptors: (Patho)physiological Relevance and Implications for Drug Discovery. *Br. J. Pharmacol.* **2000**, *130*, 1-12.

(17) Rossier, O.; Abuin, L.; Fanelli, F.; Leonardi, A.; Cotecchia, S. Inverse Agonism and Neutral Antagonism at α_{1A} and α_{1B} Adrenergic Receptor Subtypes. *Mol. Pharmacol.* **1999**, *56*, 858-866.

(18) Pauwels, P. J.; Tardif, S.; Wurch, T.; Colpaert, F. C. Facilitation of Constitutive α_{2A} Adrenoceptor Activity by Both Single Amino Acid Mutation ($Thr^{373}Lys$) and $G_{\alpha 0}$ Protein. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 654-663.
(19) Chidiac, P.; Hebert, T. E.; Valiquette, M.; Dennis, M.; Bouvier, M. Inverse Agonist Activity of β-Adrenergic Agonists. *Mol. Pharmacol.* **1994**, *45*, 490-499.
(20) Barr, A. J.; Manning, D. R. Agonist-independent Activation of G_z by the 5-$Hydroxytriptamine_{1A}$ Receptor Co-expressed in *Spodoptera Frugiperda* Cells. *J. Biol. Chem.* **1997**, *272*, 32979-32987.
(21) Smit, M. J.; Leurs, R.; Alewijnse, A. E.; Blauw, J.; Van Nieuw Amerongen, G. P.; Van de Vrede, Y.; Roovers, E.; Timmerman, H. Inverse Agonism of Histamine H_2 Antagonists Accounts for Upregulation of Spontaneously Active Histamine H_2 Receptors. *Proc. Natl. Acad. Sci.* **1996**, *93*, 6802-6807.
(22) Hall, D. A.; Strange, P. G. Evidence that Antipsychotic Drugs are Inverse Agonists at D2 Dopamine Receptors. *Br. J. Pharmacol.* **1997**, *121*, 731-736.
(23) Westphal, R. S.; Sanders-Bush, E. Reciprocal Binding Properties of 5-HT2C Receptor Agonists and Antagonists. *Mol. Pharmacol.* **1994**, *46*, 937-942.
(24) Bakker, R. A.; Wieland, K.; Timmerman, H.; Leurs, R. Constitutive Activity of the Histamine H_1 Receptor Reveals Inverse Agonism of Histamine H_1 Receptor Antagonists. *Eur. J. Pharmacol.* **2000**, *381*, R5-R7.
(25) Leurs, R.; Rodriguez Pena, M. S.; Bakker, R. A.; Alewijnse, A. E.; Timmerman, H. Constitutive Activity of G-protein Coupled Receptors and Drug Action. *Pharm. Acta Helv.* **2000**, *74*, 327-331.
(26) Morisset, S.; Rouleau, A.; Ligneau, X.; Gbahou, F.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Schwartz, J. C.; Arrang, J. M. High Constitutive Activity of Native H_3 Receptors Regulates Histamine Neurons in Brain. *Nature* **2000**, *408*, 860-864.
(27) Nwokolo, C. U.; Smith, J. T. L.; Sawyer, A. M.; Pounder, R. E. Rebound Intragastric Hyperacidity After Abrupt Withdrawal of Histamine H_2 Receptor Blockade. *Gut* **1991**, *32*, 1455-1460.
(28) Merki, H. S.; Wilder-Smith, C. H. Do Continuous Infusion of Omeprazole and Ranitidine Retain their Effect with Prolonged Dosing? *Gastroenterology* **1994**, *106*, 60-64.
(29) Alewijnse, A. E.; Smit, M. J.; Hoffmann, M.; Verzijl, D.; Timmerman, H.; Leurs, R. Constitutive Activity and Structural Instability of the Wild-Type Human H_2 Receptor. *J. Neurochem.* **1998**, *71*, 799-807.
(30) Elfellah, M. S.; Reid, J. L. Regulation of β_1- and β_2-adrenoceptors Following Chronic Treatment with β-adrenoceptor Antagonists. *Eur. J. Pharmacol.* **1989**, *173*, 85-92.
(31) Homcy, C. J.; Vatner, S. F.; Vatner, D. E. Beta Adrenergic Receptor Regulation in the Heart in Pathophysiological States: Abnormal Adrenergic Responsiveness in Cardiac Disease. *Annu. Rev. Physiol.* **1991**, *53*, 139-157.
(32) Motomura, S.; Deighton, N. M.; Zerkowski, H. R.; Doetsch, N.; Michel, M. C.; Brodde, O. E. Chronic Beta-1-adrenoceptor Antagonist Treatment Sensitizes Beta-2 Adrenoceptors, but Desensitizes M2-muscarinic Receptor in the Human Right Atrium. *Br. J. Pharmacol.* **1990**, *101*, 363-369.
(33) Yoburn, B. C.; Duttaroy, A.; Shah, S.; Davis, T. Opioid Antagonist-induced Receptor Upregulation: Effects of Concurrent Agonist Administration. *Brain Res.* **1994**, *33*, 237-240.
(34) Nijenhuis, W. A.; Oosterom, J.; Adan, R. A. AgRP (83-132) acts as an Inverse Agonist on the Human-melanocortin-4 Receptor. *Mol. Endocrinol.* **2001**, *15*, 164-171.
(35) Askell-Luevano, C.; Monck, E. K. Agouti-related Protein Functions as an Inverse Agonist at a Constitutively Active Brain Melanocortin-4 Receptor. *Regul. Pept.* **2001**, *99*, 1-7.
(36) Adan, R. A. H.; Kas, M. J. H. Inverse Agonism Gains Weight. *Trends Pharm. Sci.* **2003**, *24*, 315-321.
(37) Han, M.; Smith, S. O.; Sakmar, T. P. Constitutive Activity of Opsin by Mutation of Methionine 257 on Transmembrane Helix 6. *Biochemistry* **1998**, *37*, 8253-8261.
(38) Geras-Raaka, E.; Varma, A.; Ho, H.; Clark-Lewis, I.; Gershengorn, M. C. Human Interferon-gamma-inducible Protein 10 (IP-10) Inhivits Constitutive Signalling of

Kaposi's Sarcoma-associated Herpesvirus G Protein-coupled Receptor. *J. Exp. Med.* **1998**, *188*, 405-408.

(39) Rosenkilde, M. M.; Kledal, T. N.; Braüner-Osborne, H.; Schwartz, T. W. Agonists and Inverse Agonists for the Herpesvirus 8-Encoded Constitutively Active Seven Transmembrane Oncogene Product, ORF-74. *J. Biol. Chem.* **1999**, *274*, 959-961.

(40) Casarosa, P.; Bakker, R. A.; Verzijl, D.; Navis, M.; Timmerman, H.; Leurs, R.; Smit, M. J. Constitutive Signaling of the Human Cytomegalovirus-encoded Chemokine Receptor US28. *J. Biol. Chem.* **2001**, *276*, 1133-1137.

(41) Pauwels, P. J.; Wurch, T. Review: Amino Acid Domains Involved in Constitutive Activation of G-protein-coupled Receptors. *Mol. Neurobiol.* **1998**, *17*, 109-135.

(42) Shenker, A.; Laue, L.; Kosugi, S.; Merendino, J. J.; Minegishi, T.; Cutler, G. B. A Constitutively Activating Mutation of the Luteinizing Hormone Receptor in Familial Male Precocious Puberty. *Nature* **1993**, *365*, 652.

(43) Robinson, P. R.; Cohen, G. B.; Zhukovsky, E. A.; Oprian, D. D. Constitutively Active Mutants of Rhodopsin. *Neuron* **1992**, *9*, 719-725.

(44) Parma, J.; Duprez, L.; Van Sande, J.; Cochaux, P.; Gervy, C.; Mockel, J.; Dumont, J.; Vassart, G. Somatic Mutations in the Thyrotropin Receptor Gene Cause Hyperfunctioning Thyroid Adenomas. *Nature* **1993**, *365*, 649-651.

(45) Robbins, L. S.; Nadeau, J. H.; Johnson, K. R.; Kelly, M. A.; Roselli Rehfuss, L.; Baack, E.; Mountjoy, K. G.; Cone, R. D. Pigmentation Phenotypes of Variant Extension Locus Alleles Result from Point Mutation that Alter MSH Receptor Function. *Cell* **1993**, *72*, 827-834.

(46) Barger, G.; Dale, H. H. Chemical Structure and Sympathomimetic Actions of Histamine. *J. Physiol. (London)* **1910**, *41*, 19-59.

(47) Dale, H. H.; Laidlaw, P. P. The Physiological Action of b-imidazolylethylamine. *J. Physiol. (London)* **1910**, *41*, 418-444.

(48) Yamashita, M.; Fukui, H.; Sugama, K.; Horio, Y.; Ito, S.; Mizuguchi, H.; Wada, H. Expression Cloning of a cDNA Encoding the Bovine Histamine H_1 Receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11515-11519.

(49) Gantz, I.; Schaffer, L.; Del Valle, J.; Logsdon, C.; Campbell, V.; Uhler, M.; Yamada, T. Molecular Cloning of a Gene Encoding the Histamine H_2 Receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 429-433.

(50) Lovenberg, T. W.; Roland, B. L.; Wilson, S. J.; Jiang, X.; Pyati, J.; Huvar, A.; Jackson, M. R.; Erlander, M. G. Cloning and Functional Expression of the Human Histamine H_3 Receptor. *Mol. Pharmacol.* **1999**, *55*, 1101-1107.

(51) Oda, T.; Morikawa, N.; Saito, Y.; Masuho, Y.; Matsumoto, S. I. Molecular Cloning and Characterization of a Novel Type of Histamine Receptor Preferentially Expressed in Leukocytes. *J. Biol. Chem.* **2000**, *275*, 36781-36786.

(52) Zhang, M. Q.; Leurs, R.; Timmerman, H. Histamine H_1-Receptor Antagonists. *Burger's Medicinal Chemistry and Drug Discovery*; Wiley: New York, 1997; pp 495-559.

(53) Ash, A. S. F.; Schild, H. O. Receptors Mediating Some Actions of Histamine. *Br. J. Pharmacol.* **1966**, *27*, 427-439.

(54) Black, J. W.; Duncan, W. A. M.; Durant, G. J.; Ganellin, C. R.; Parsons, M. E. Definition and Antagonism of Histamine H_2 Receptors. *Nature* **1972**, *236*, 385-390.

(55) Arrang, J. M.; Garbag, M.; Schwartz, J. C. Auto-inhibition of Brain Histamine Release Mediated by a Novel Class (H_3) of Histamine Receptors. *Nature Lond.* **1983**, *302*, 832-837.

(56) Leurs, R.; Timmerman, H. The Histamine H_3 Receptor: A Target for Developing New Drugs. *Prog. Drug Res.* **1992**, *39*, 127-165.

(57) Leurs, R.; Blandina, P.; Tedford, C.; Timmerman, H. Therapeutic Potential of Histamine H_3 Receptor Agonists and Antagonists. *Trends Pharm. Sci.* **1998**, *19*, 177-183.

(58) Raible, D. G.; Lenahan, T.; Fayvilevich, Y.; Kosinski, R.; Schulman, E. S. Pharmacological Characterization of a Novel Histamine Receptor on Human Eosinophils. *Am. J. Respir. Crit. Care Med.* **1994**, *149*, 1506-1511.

(59) Liu, C.; Ma, X. J.; Jiang, X.; Wilson, S. J.; Hofstra, C. L.; Blevitt, J.; Pyati, J.; Li, X.; Chai, W.; Carruthers, N.; Lovenberg, T. W. Cloning and Pharmacological

Characterization of a Fourth Histamine Receptor (H_4) Expressed in Bone Marrow. *Mol. Pharmacol.* **2001**, *59*, 420-426.

(60) Nakamura, T.; Itadani, H.; Hikada, Y.; Ohta, M.; Tanaka, K. Molecular Cloning and Characterization of a New Histamine Receptor, *HH4R*. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 615-620.

(61) Morse, K. L.; Behan, J.; Laz, T. M.; West, R. E. J.; Greenfeder, S. A.; Anthes, J. C.; Umland, S.; Wan, Y.; Hipkin, R. W.; Gonsiorek, W.; Shin, N.; Gustafson, E. L.; Qiao, X.; Wang, S.; Hedrick, J. A.; Greene, J.; Bayne, M.; Monsma, F. J. J. Cloning and Characterization of a Novel Human Histamine Receptor. *J. Pharm. Exp. Ther.* **2001**, *296*, 1058-1066.

(62) Zhu, Y.; Michalovich, D.; Wu, H. L.; Tan, K. B.; Dytko, G. M.; Mannan, I. J.; Boyce, R.; Altson, J.; Tierney, L. A.; Li, X.; Herrity, N. C.; Vawter, L.; Sarau, H. M.; Ames, R. S.; Davenport, C. M.; Hieble, J. P.; Wilson, S.; Bergsma, D. J.; Fitzgerald, L. R. Cloning, Expression, and Pharmacological Characterization of a Novel Human Histamine Receptor. *Mol. Pharmacol.* **2001**, *59*, 434-441.

(63) Nguyen, T.; Shapiro, D. A.; George, S. R.; Setola, V.; Lee, D. K.; Cheng, R.; Rauser, L.; Lee, S. P.; Lynch, K. R.; Roth, B. L.; O'Dowd, B. F. Discovery of a Novel Member of the Histamine Receptor Family. *Mol. Pharmacol.* **2001**, *59*, 427-433.

(64) Hill, S. J. Distribution, Properties and Functional Characteristics of Three Classes of Histamine Receptor. *Pharmacol. Rev.* **1990**, *42*, 45-83.

(65) Schwartz, J. C.; Arrang, J. M.; Garbarg, M.; Pollard, H.; Ruat, M. Histaminergic Transmission in the Mammalian Brain. *Physiol. Rev.* **1991**, *71*, 1-51.

(66) Fujimoto, K.; Horio, Y.; Sugama, K.; Ito, S.; Liu, Y. Q.; Fukui, H. Genomic Cloning of the Rat Histamine H_1 Receptor. *Biochem. Biophys. Res. Commun.* **1993**, *190*, 294-301.

(67) Horio, Y.; Mori, Y.; Higuchi, I.; Fujimoto, K.; Ito, S.; Fukui, H. Molecular Cloning of the Guinea-pig Histamine H_1-receptor Gene. *J. Biochem.* **1993**, *114*, 408-414.

(68) Traiffort, E.; Leurs, R.; Arrang, J. M.; Tardivel-Lacombe, J.; Diaz, J.; Schwartz, J.-C.; Ruat, M. Guinea Pig Histamine H_1 Receptor. I. Gene Cloning, Characterization, and Tissue Expression Revealed by In Situ Hydridization. *J. Neurochem.* **1994**, *62*, 507-518.

(69) Inoue, I.; Taniuchi, I.; Kitamura, D.; Jenkins, N. A.; Gilbert, D. J.; Copeland, N. G.; Watanabe, T. Characteristics of the Mouse Genomic Histamine H_1-receptor Gene. *Genomics* **1996**, *36*, 178-181.

(70) De Backer, M. D.; Gommeren, W.; Moereels, H.; Nobels, G.; Van Gompel, G.; Leysen, J. E.; Luyten, W. H. M. L. Genomic Cloning, Heterelogous Expression and Pharmacological Characterization of a Human Histamine H_1-receptor. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 1601-1608.

(71) Fukui, H.; Fujimoto, K.; Mizuguchi, H.; Sakamoto, K.; Horio, Y.; Takai, S.; Yamada, T.; Ito, S. Molecular Cloning of the Human H_1-receptor Gene. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 894-901.

(72) Moguilevsky, N.; Varsalona, F.; Noyer, M.; Gillard, M.; Guillaume, J. P.; Garcia, L.; Szpirer, J.; Bollen, A. Stable Expression of Human H_1-histamine-receptor cDNA in Chinese Hamster Ovary Cells: Pharmacological Characterization of the Protein, Tissue Distribution of Messenger RNA and Chromosomal Localization of the Gene. *Eur. J. Biochem.* **1994**, *224*, 489-495.

(73) Leurs, R.; Traiffort, E.; Arrang, J.-M.; Tardivel-Lacombe, J.; Ruat, M.; Schwartz, J.-C. Guinea Pig Histamine H1 Receptor. II. Stable Expression in Chinese Hamster Ovary Cells Reveal the Interaction with Three Major Signal Transduction Pathways. *J. Neurochem.* **1994**, *62*, 519-527.

(74) Hill, S. J.; Donaldson, J. The H_1-receptor and Inositol Phospholipid Hydrolysis. *The Histamine Receptor*; Wiley-Liss: New York, 1992; pp 109-128.

(75) Leurs, R.; Smit, M. J.; Timmerman, H. Molecular Pharmacological Aspects of Histamine Receptors. *Pharmacol. Ther.* **1995**, *66*, 413-463.

(76) Schmidt, H. H. T. W.; Zernikow, B.; Baeblich, S.; Bohme, E. Basal and Stimulated Formation and Release of L-arginine-derived Nitrogen Oxides from Cultured Endothelial Cells. *J. Pharmacol. Exp. Ther.* **1990**, *254*, 591-597.

(77) Leurs, R.; Brozius, M. M.; Jansen, W.; Bast, A.; Timmerman, H. Histamine H_1-receptor Mediated Cyclic GMP Production in Guinea-pig Lung Tissue is an L-arginine-dependent Process. *Biochem. Pharmacol.* **1991**, *42*, 271-277.

(78) Carter, T. D.; Hallam, T. J.; Cusack, N. J.; Pearson, J. D. Regulation of P_{2y}-purinoceptor-mediated Prostacyclin Release from Human Endothelial Cells by Cytoplasmic Calcium Concentration. *Br. J. Pharmacol.* **1988**, *95*, 1181-1190.

(79) Resink, T. J.; Grigorian, G. Y.; Moldabaeva, A. K.; Danilov, S. M.; Buhler, F. R. Histamine-induced Phosphoinositide Metabolism in Cultured Human Umbilical Vein Endothelial Cells: Association with Tromboxane and Prostacyclic Release. *Biochem. Biophys. Res. Commun.* **1987**, *144*, 438-446.

(80) Palacios, J. M.; Garbarg, M.; Barbin, G.; Schwartz, J.-C. Pharmacological Characterization of Histamine Receptors Mediating the Stimulation of cAMP Accumulation in Slices from Guinea-pig Hippocampus. *Mol. Pharmacol.* **1978**, *14*, 971-982.

(81) Al-Gadi, M.; Hill, S. J. Characterization of Histamine Receptors Mediating the Stimulation of Cyclic AMP Accumulation in Rabbit Cerebral Cortical Slices. *Br. J. Pharmacol.* **1985**, *85*, 877-888.

(82) Aoki, Y.; Qiu, D.; Zhao, G. H.; Kao, P. N. Leukotriene B_4 Mediates Histamine Induction of NF-κB and IL-8 in Human Bronchial Epitelial Cells. *Am. J. Physiol.* **1998**, *274*, L1030-L1039.

(83) Hu, Q.; Deshpande, S.; Irani, K.; Ziegelstein, R. C. $[Ca^{2+}]_i$ Oscillation Frequency Regulates Agonist-stimulated NF-κB Transcriptional Activity. *J. Biol. Chem.* **1999**, *274*, 33995-33998.

(84) Bakker, R. A.; Schoonus, S. B. J.; Smit, M. J.; Timmerman, H.; Leurs, R. Histamine H_1-Receptor Activation of Nuclear Factor-κB: Roles for Gβγ- and $G\alpha_{q/11}$-Subunits in Constitutive and Agonist-Mediated Signaling. *Mol. Pharmacol.* **2001**, *60*, 1133-1142.

(85) Leurs, R.; Church, M. K.; Taglialatela, M. H_1-antihistamines: Inverse Agonism, Anti-inflammatory Actions and Cardiac Effects. *Clin. Exp. All.* **2002**, *32*, 489-498.

(86) Karin, M.; Ben-Neriah, Y. Phosphorylation Meets Ubiquitination: the Control of NF-κB Activity. *Annu. Rev. Immunol.* **2000**, *18*, 621-663.

(87) Iriyoshi, N.; Takeuchi, K.; Yuta, A.; Ukai, K.; Sakakura, Y. Increased Expression of Histamine H_1 Receptor mRNA in Allergic Rhinitis. *Clin. Exp. Allergy* **1996**, *26*, 376-385.

(88) Hamano, N.; Terada, N.; Maesako, K.; Ikeda, T.; Fukuda, S.; Wakita, J.; Yamashita, T.; Konno, A. Expression of Histamine Receptors in Nasal Epithelial Cells and Endothelial Cells-The Effects of Sex Hormones. *Int. Arch. Allergy Immunol.* **1998**, *115*, 220-227.

(89) Lintunen, M.; Sallmen, T.; Karlstedt, K.; Fukui, H.; Eriksson, K. S.; Panula, P. Postnatal Expression of H_1-receptor mRNA in the Rat Brain: Correlation to l-Histidine Decarboxylase Expresion and Local Upregulation in Limbic Seizure. *Eur. J. Neurosci.* **1998**, *10*, 2287-2301.

(90) Iinuma, K.; Yokoyama, H.; Otsuki, T.; Yanai, K.; Watanabe, T.; Ido, T.; Itoh, M. Histamine H_1-receptors in Complex Partial Seizures. *Lancet* **1993**, *341*, 238.

(91) Chiba, Y.; Saitoh, N.; Matsuo, K.; Misawa, M. Elevated Nasal Mucosal G Protein Levels and Histamine Receptor Affinity in a Guinea Pig Model of Nasal Hyperresponsiveness. *Int. Arch. Allergy Immunol.* **2002**, *127*, 285-293.

(92) Govoni, M.; Bakker, R. A.; van de Wetering, I.; Smit, M. J.; Menge, W. M. B. P.; Timmerman, H.; Elz, S.; Schunack, W.; Leurs, R. Synthesis and Pharmacological Identification of Neutral Histamine H_1-Receptor Antagonists. *J. Med. Chem.* **2003**, *46*, 5812-5824.

(93) Arrang, J. M.; Garbarg, M.; Quach, T. T.; Tuong, M. D. T.; Yeramian, E.; Schwartz, J. C. Actions of Betahistine at Histamine Receptors in the Brain. *Eur. J. Pharmacol.* **1985**, *111*, 73-84.

(94) Scherkl, R.; Hashem, A.; Frey, H. H. Importance of Histamine for Seizure Susceptibility. *Agents Actions Suppl.* **1991**, *33*, 85-89.

(95) Yokoyama, H.; Onodera, K.; Iinuma, K.; Watanabe, T. 2-Thiazolylethylamine, a Selective Histamine H_1 Agonist, Decreases Seizure Susceptibility in Mice. *Pharmacol. Biochem. Behav.* **1994**, *47*, 503-507.

(96) Tuomisto, L.; Tacke, U. Is Histamine an Anticonvulsive Inhibitory Transmitter? *Neuropharmacology* **1986**, *25*, 955-958.
(97) Yokoyama, H.; Onodera, K.; Maeyama, K.; Yanai, K.; Iinuma, K.; Tuomisto, L.; Watanabe, T. Histamine Levels and Clonic Convulsions of Electrically-induced Seizure in Mice: the Effects of Alpha-fluoromethylhistidine and Metoprine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1992**, *346*, 40-45.
(98) Malmberg-Aiello, P.; Ipponi, A.; Bartolini, A.; Schunack, W. Antiamnestic Effect of Metoprine and of Selective Histamine H_1 Receptor Agonists in a Modified Mouse Passive Avoidance Test. *Neurosci. Lett.* **2000**, *288*, 1-4.
(99) Malmberg-Aiello, P.; Lamberti, C.; Ipponi, A.; Bartolini, A.; Schunack, W. Evidence for Hypernociception Induction Following Histamine H_1 Receptor Activation in Rodents. *Life Sci.* **1998**, *63*, 463-476.
(100) Tashiro, M.; Mochizuki, H.; Iwabuchi, K.; Sakurada, Y.; Itoh, M.; Watanabe, T.; Yanai, K. Roles of Histamine in Regulation of Arousal and Cognition: Functional Neuroimaging of Histamine H_1 Receptors in Human Brain. *Life Sci.* **2002**, *72*, 409-414.
(101) Hill, S. J.; Ganellin, C. R.; Timmerman, H.; Schwartz, J. C.; Shankley, N. P.; Young, J. M.; Schunack, W.; Levi, R.; Haas, H. L. International Union of Pharmacology. XIII. Classification of Histamine Receptors. *Pharmacol. Rev.* **1997**, *49*, 253-278.
(102) Van der Goot, H.; Timmerman, H. Selective Ligands as Tools to Study Histamine Receptors. *Eur. J. Med. Chem.* **2000**, *35*, 5-20.
(103) Zingel, V.; Leschke, C.; Schunack, W. Developments in Histamine H_1-receptor Agonists. *Prog. Drug. Res.* **1995**, *44*, 49-85.
(104) Elz, S.; Kramer, K.; Pertz, H. H.; Detert, H.; Ter Laak, A. M.; Kühne, R.; Schunack, W. Histaprodifens: Synthesis, Pharmacological in Vitro Evaluation, and Molecular Modeling of a New Class of Highly Active and Selective Histamine H_1-Receptor Agonists. *J. Med. Chem.* **2000**, *43*, 1071-1084.
(105) Malinowska, B.; Piszcz, J.; Schlicker, E.; Kramer, K.; Elz, S.; Schunack, W. Histaprodifen, Methylhistaprodifen, and Dimethylhistaprodifen are Potent H_1-receptor Agonists in the Pithed and Anaesthetized Rat. *Naunyn-Schmiedeberg's Arch Pharmacol* **1999**, *359*, 11-16.
(106) Bruysters, M.; Pertz, H. H.; Teunissen, A.; Bakker, R. A.; Gillard, M.; Chatelain, P.; Schunack, W.; Timmerman, H.; Leurs, R. Mutational Analysis of the Histamine H_1 Receptor Binding Pocket of Histaprodifens. *Eur. J. Pharmacol.* **2004**, *487*, 55-63.
(107) Seifert, R.; Wenzel-Seifert, K.; Bürckstümmer, T.; Pertz, H. H.; Schunack, W.; Dove, S.; Buschauer, A.; Elz, S. Multiple Differences in Agonist and Antagonist Pharmacology between Human and Guinea Pig Histamine H_1-Receptor. *J. Pharmacol. Exp.Ther.* **2003**, *305*, 1104-1115.
(108) Bakker, R. A.; Weiner, D. M.; Ter Laak, A. M.; Beuming, T.; Zuiderveld, O. P.; Edelbroek, M.; U., H.; Timmerman, H.; Brann, M. R.; Leurs, R. 8R-Lisuride is a Potent Stereospecific Histamine H_1-Receptor Partial Agonist. *Mol. Pharmacol.* **2004**, *65*, 538-549.
(109) Sadek, B.; Elz, S.; Pertz, H. H.; Stark, H.; Schunack, W. Unexpected Partial H_1-Receptor Agonism of Imidazole-type Histamine H_3-Receptor Antagonists Lacking a Basic Side Chain. *Inflamm. Res.* **2004**, *53*, S109-S115.
(110) Ison, R. R.; Franks, F. M.; Soh, K. S. The Binding of Conformationally Restricted Antihistamines to Histamine Receptors. *J. Pharm. Pharmacol.* **1973**, *25*, 887-894.
(111) Laduron, P. M.; Janssen, P. F. M.; Gommeren, W.; Leysen, J. E. In Vitro and In Vivo Binding Characteristics of a New Long-acting Histamine H_1-antagonist, Astemizole. *Mol. Pharmacol.* **1982**, *21*, 294-300.
(112) Timmerman, H. Factors Involved in the Incidence of Central Nervous System Effects of H_1 Blockers. *Therapeutics Index of Antihistamines*; Hogrefe and Huber: Lewinston, 1992; pp 19-31.
(113) Rose, C.; Quach, T. T.; Lorens, C.; Schwartz, J.-C. Realtionship Between Occupation of Cerebral H_1-receptors and Sedative Properties of Antihistamines. *Arzneim.-Forsch.* **1982**, *32 (Suppl. 11)*, 1171-1173.
(114) Markham, A.; Wagstaff, A. J. Fexofenadine. *Drugs* **1998**, *55*, 269-274.

(115) Ahn, H. S.; Barnett, A. Selective Displacement of ^{3}H-mepyramine from Peripheral vs Central Nervous System Receptors by Loratadine, a Non-sedative Antihistamine. *Eur. J. Pharmacol.* **1986**, *127*, 153-155.
(116) Monti, J. M. Involvement of Histamine in the Control of the Waking State. *Life Sci.* **1993**, *53*, 1331-1338.
(117) Taglialatela, M.; Castaldo, P.; Pannaccione, A.; Giorgio, G.; Genovese, A.; Marone, G.; Annunziato, L. Cardian Ion Channels and Antihistamines: Possible Mechanisms of Cardiotoxicity. *Clin. Exp. Allergy* **1999**, *29*, 182-189.
(118) Yap, Y. G.; Camm, A. J. Potential Cardiac Toxicity of H_1-antihistamines. *Clin. Allergy Immunol.* **2002**, *17*, 389-419.
(119) DuBuske, L. M. Second-generation Antihistamines: The Risk of Ventricular Arrhythmias. *Clin. Ther.* **1999**, *21*, 281-295.
(120) Hegstrand, L. R.; Kanof, P. D.; Greengard, P. Histamine-sensitive Adenylate Cyclase in Mammalian Brain. *Nature (Lond.)* **1976**, *260*, 163-165.
(121) Soll, A. H.; Wolin, A. Histamine and Cyclic AMP in Isolated Canine Parietal Cells. *Am. J. Physiol.* **1979**, *237*, E444-E450.
(122) Gespach, C.; Bouhours, D.; Bouhours, J. F.; Rosselin, G. Histamine Interaction on Surface Recognition Sites of H_2-type in Parietal and Non-parietal Cells Isolated from Guinea-pig Stomach. *FEBS Lett.* **1982**, *149*, 85-90.
(123) Johnson, C. L.; Weinstein, H.; Green, J. P. Studies on Histamine H_2 Receptors Coupled to Cardiac Adenylate Cyclase: Blockade by H_2 and H_1 Receptor Antagonists. *Mol. Pharmacol.* **1979**, *16*, 417-428.
(124) Kanof, P. D.; Greengard, P. Pharmacological Properties of Histamine-sensitive Adenylate Cyclase from Guinea-pig Cardiac Ventricular Muscle. *Mol. Pharmacol.* **1979**, *15*, 445-461.
(125) Hattori, Y.; Kimura, S.; Fujii, S.; Kanno, M. Effects of Mechanical Performance and Biochemical and Electrical Activity in the Heart of Monkeys (Mocaca Fuscata). *Eur. J. Pharmacol.* **1983**, *91*, 11-19.
(126) Levi, R. C.; Alloatti, G. Histamine Modulates Calcium Current in Guinea-pig Ventricular Myocytes. *J. Pharmacol. Exp. Ther.* **1988**, *246*, 377-383.
(127) Reinhardt, D.; Ritter, E. Hypothermia-induced Potentiation of Histamine H_2-receptor-mediated Relaxation and Cyclic AMP Increase in Isolated Mesentery Artery of the Rabbit. *Agents Actions* **1979**, *9*, 9-14.
(128) Haas, H. L.; Konnerth, A. Histamine and Noradrenaline Decrease Calcium-activated Potassium Conductance in Hippocampal Pyramidal Cells. *Nature (Lond.)* **1983**, *302*, 432-434.
(129) Haas, H. L.; Greene, R. W.; Heimrich, B.; Xie, X. LTP in Slices from Human Hippocampus. *Synaptic Plasticity in the Hippocampus*; Springer: Berlin, 1988; pp 77-80.
(130) Ruat, M.; Traiffort, E.; Arrang, J.-M.; Leurs, R.; Schwartz, J.-C. Cloning and Tissue Expression of a Rat Histamine H_2-receptor Gene. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 1470-1478.
(131) Gantz, I.; Munzert, G.; Tashiro, T.; Schäffer, M.; Wang, L.; Yamada, T. Molecular Cloning of the Human Histamine H_2 Receptor. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 1386-1392.
(132) Traiffort, E.; Vizuete, M. L.; Tardivel-Lacombe, J.; Souile, E.; Schwartz, J.-C.; Ruat, M. The Guinea-pig Histamine H_2 Receptor-gene Cloning, Tissue Expression and Chromosomal Localization of its Human Counterpart. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 570-577.
(133) Kobayashi, T.; Inove, I.; Jenkins, N. A.; Gilbert, D. J.; Copeland, N. G.; Watanabe, T. Cloning, RNA Expression and Chromosomal Location of a Mouse Histamine H_2-receptor Gene. *Genomics* **1996**, *37*, 390-394.
(134) Johnson, C. L. Histamine Receptors and Cyclic Nucleotides. *Pharmacology of Histamine Receptors*; Wright: Bristol, England, 1982; pp 146-216.
(135) Chew, C. S. Differential Effects of Extracellular Calcium Removal and Non-specific Effects of Ca^{2+} Antagonists on Acid Secretory Activity in Isolated Gastric Glands. *Biochem. Biophys. Acta* **1985**, *846*, 370-378.

(136) Mitsuhashi, M.; Mitsuhashi, T.; Payan, D. G. Multiple Signalling Pathways of Histamine H_2 Receptors: Identification of an H_2 Receptor Dependent Ca^{2+} Mobilization Pathway in Human HL-60 Promyelitic Leukemia Cells. *J. Biol. Chem.* **1989**, *264*, 18356-18362.
(137) Del Valle, J.; Wang, L.; Gantz, I.; Yamada, T. Characterization of H_2 Histamine Receptor: Linkage to Both Adenylate Cyclase and $[Ca^{2+}]_i$ Signaling System. *Am. J. Physiol.* **1992**, *263*, G967-G972.
(138) Durant, G. J.; Duncan, W. A. M.; Ganellin, C. R.; Parsons, M. E.; Blakemore, R. C.; Rasmussen, A. C. Impromidine (SKandF 92676) is a very Potent and Specific Agonist for Histamine H_2-receptors. *Nature (Lond.)* **1978**, *276*, 403-405.
(139) Elz, S.; Gerhard, G.; Schunack, W. Histamine Analogues: 32nd Communication-Synthesis and Pharmacology of Sopromidine, a Potent and Stereoselective Isomer of the Achiral H_2-agonist Impromidine. *Eur. J. Med. Chem.* **1989**, *24*, 259-262.
(140) Sellier, C.; Elz, S.; Buschauer, A.; Schunack, W. The Effect of Lipophilic Substituents on the H_2-histaminergic Activity of Some Close Analogues of Impromidine. *Arch. Pharm. (Weinheim)* **1992**, *325*, 471-476.
(141) Parsons, M. E.; Owen, D. A. A.; Ganellin, C. R.; Durant, G. J. Dimaprit -[S-[3(N,N-dimethylamino)propyl]isothiourea]- A Highly Specific Histamine H_2 Receptor Agonist. *Agents Actions* **1977**, *7*, 31-37.
(142) Eriks, J. C.; Van der Goot, H.; Sterk, G. J.; Timmerman, H. Histamine H_2-receptor Agonists. Synthesis, In Vitro Pharmacology, and Qualitative Structure-Activity Relationships of Substituted 4- and 5-(2-aminoethyl)thiazoles. *J. Med. Chem.* **1992**, *35*, 3239-3246.
(143) Brimblecombe, R. W.; Duncan, W. A. M.; Durant, G. J.; Emmett, J. C.; Ganellin, C. R.; Parsons, M. E. Cimetidine: a Non-thiourea H_2-receptor Antagonist. *J. Int. Med. Res.* **1975**, *3*, 86-92.
(144) Black, J. W.; Durant, G. J.; Emmett, J. C.; Ganellin, C. R. Sulphur-methylene Isosterism in the Development of Metiamide, a New Histamine H_2-receptor Antagonist. *Nature (Lond.)* **1974**, *248*, 65-67.
(145) Bradshaw, J.; Brittain, R. T.; Clitherow, J. W.; Daly, M. J.; Jack, D.; Price, B. J.; Stables, R. Ranitidine, a New Potent Selective H_2-receptor Antagonist. (Abstract). *Br. J. Pharmacol.* **1979**, *66*, 464P.
(146) Yellin, T. O.; Buck, S. H.; Gilman, D. J.; Jones, D. F.; Wardleworth, J. M. ICI 125,211 a New Gastric Antisecretory Agent Acting on Histamine H_2-receptors. *Life Sci.* **1979**, *25*, 2001-2009.
(147) Hirshfeld, J.; Buschauer, A.; Elz, S.; Schunack, W.; Ruat, M.; Traiffort, E.; Schwartz, J.-C. Iodoaminopotentidine and Related Compounds: a New Class of Ligands with High Affinity and Selectivity for the Histamine H_2 Receptor. *J. Med. Chem.* **1992**, *35*, 2231-2238.
(148) Arrang, J. M.; Garbarg, M.; Schwartz, J. C. Autoregulation of Histamine Release in Brain by Presynaptic H_3 Receptors. *Neuroscience* **1985**, *15*, 553-562.
(149) Arrang, J. M.; Garbarg, M.; Schwartz, J. C. Autoinhibition of Histamine Synthesis Mediated by Presynaptic H_3 Receptors. *Neuroscience* **1987**, *23*, 149-157.
(150) Clapham, J.; Kilpatrick, G. J. Histamine H_3 Receptors Modulate the Release of $[^3H]$-Acetylcholine from Slices of Rat Entorhinal Cortex: Evidence for the Possible Existence of H_3 Receptor Sybtypes. *Br. J. Pharmacol.* **1992**, *107*, 919-923.
(151) Yokotani, K.; Murakami, Y.; Okada, S.; Wang, M.; Nakamura, K. Histamine H3 Receptor-mediated Inhibition of Endogenous Acetylcholine Release from the Isolated, Vascularly Perfused Rat Stomach. *Eur. J. Pharmacol.* **2000**, *392*, 23-29.
(152) Schlicker, E.; Fink, K.; Detzner, M.; Gothert, M. Histamine Inhibits Dopamine Release in the Mouse Striatum via Presynaptic H_3 Receptors. *J. Neural Transm. Gen. Sect.* **1993**, *93*, 1-10.
(153) Schlicker, E.; Schunack, W.; Gothert, M. Histamine H_3 Receptor-mediated Inhibition of Noradrenaline Release in Pig Retina Discs. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1990**, *342*, 497-501.

(154) Schlicker, E.; Betz, R.; Gothert, M. Histamine H_3 Receptor-mediated Inhibition of Serotonin Release in the Rat Brain Cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1988**, *337*, 588-590.
(155) Brown, R. E.; Reymann, K. G. Histamine H_3 Receptor-mediated Depression of Synaptic Transmission in the Dentate Gyrus of the Rat In Vitro. *J. Physiol.* **1996**, *496*, 175-184.
(156) Matsubara, T.; Moskowitz, M. A.; Huang, Z. UK 14,304, R(-)-α-methylhistamine, and Sms 201-995 Block Plasma Protein Leakage within Dura Mater by Prejunctional Mechanisms. *Eur. J. Pharmacol.* **1992**, *224*, 145-150.
(157) Lovenberg, T. W.; Pyati, J.; Chang, H.; Wilson, S. J.; Erlander, M. G. Cloning of Rat Histamine H_3 Receptor Reveal Distinct Species Pharmacological Profiles. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 771-778.
(158) Tardivel-Lacombe, J.; Rouleau, A.; Heron, A.; Morisset, S.; Pillot, C.; Cochois, V.; Schwartz, J. C.; Arrang, J. M. Cloning and Cerebral Expression of the Guinea Pig Histamine H_3 Receptor: Evidence for two Isoforms. *NeuroReport* **2000**, *11*, 755-759.
(159) Yao, B. B.; Sharma, R.; Cassar, S.; Esbenshade, T. A.; Hancock, A. A. Cloning and Pharmacological Characterization of the Monkey Histamine H_3 Receptor. *Eur. J. Pharm.* **2003**, *482*, 49-60.
(160) Chen, J.; Liu, C.; Lovenberg, T. W. Molecular and Pharmacological Characterization of the Mouse Histamine H_3 Receptor. *Eur. J. Pharm.* **2003**, *467*, 57-65.
(161) Drutel, G.; Peitsaro, N.; Karlstedt, K.; Wieland, K.; Smit, M. J.; Timmerman, H.; Panula, P.; Leurs, R. Identification of Rat H_3 Receptor Isoforms with Different Brain Expression and Signaling Properties. *Mol. Pharmacol.* **2001**, *1*, 1-8.
(162) Cogé, F.; Guenin, S. P.; Audinot, V.; Renouard-Try, A.; Beauverger, P.; Macia, C.; Ouvry, C.; Nagel, N.; Boutin, J. A.; Galizzi, J. P. Genomic Organization and Characterization of Splice Variants of the Human Histamine H_3 Receptor. *Biochem. J.* **2001**, *355*, 279-288.
(163) Wellendorph, P.; Goodman, M. W.; Burstein, E. S.; Nash, N. R.; Brann, M. R.; Weiner, D. M. Molecular Cloning and Pharmacology of Functionally Distinct Isoforms of the Human Histamine H_3 Receptor. *Neuropharmacology* **2002**, *42*, 929-940.
(164) Rouleau, A.; Héron, A.; Cochois, V.; Pillot, C.; Schwartz, J.-C.; Arrang, J.-M. Cloning and Expression of the Mouse Histamine H_3 Receptor: Evidence for Multiple Isoforms. *J. Neurochem.* **2004**, *90*, 1331-1338.
(165) Stark, H.; Sippl, W.; Ligneau, X.; Arrang, J. M.; Ganellin, C. R.; Schwartz, J. C.; Schunack, W. Different Antagonist Binding Properties of Human and Rat Histamine H_3 Receptor. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 951-954.
(166) Arrang, J. M.; Garbag, M.; Lancelot, J. C.; Lecomte, J. M.; Pollard, H.; Robba, M.; Schunack, W.; Schwartz, J. C. Highly Potent and Selective Ligands for Histamine H_3-Receptors. *Nature* **1987**, *327*, 117-123.
(167) Ligneau, X.; Morisset, S.; Tardivel-Lacombe, J.; Gbahou, F.; Ganellin, C. R.; Stark, H.; Schunack, W.; Schwartz, J. C.; Arrang, J. M. Distinct Pharmacology of Rat and Human Histamine H_3 Receptors: Role of Two Amino Acids in the Third Transmembrane Domain. *Br. J. Pharmacol.* **2000**, *131*, 1247-1250.
(168) Ireland-Denny, L.; Parihar, A. S.; Miller, T. R.; Kang, C. H.; Krueger, K. M.; Esbenshade, T. A.; Hancock, A. A. Species-related Pharmacological Heterogeneity of Histamine H_3 Receptors. *Eur. J. Pharmacol.* **2001**, *433*, 141-150.
(169) Gomez-Ramirez, J.; Ortiz, J.; Blanco, I. Presynaptic H_3 Autoreceptor Modulate Histamine Synthesis through cAMP Pathway. *Mol. Pharmacol.* **2002**, *61*, 239-245.
(170) Silver, R. B.; Mackins, C. J.; Smith, N. C. E.; Koritchneva, I. L.; Lefkowitz, K.; Lovenberg, T. W.; Levi, R. Coupling of Histamine H_3 Receptors to Neuronal Na^+/H^+ Exchange: a Novel Protective Mechanism in Myocardial Ischemia. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2855-2859.
(171) Wieland, K.; Bongers, G.; Yamamoto, Y.; Hashimoto, T.; Yamatodani, A.; Menge, W. M. P. B.; Timmerman, H.; Lovenberg, T. W.; Leurs, R. Constitutive Activity of Histamine H_3 Receptors Stably Expressed in SK-N-MC Cells: Display of Agonism and Inverse Agonism by H_3 Antagonists. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 908-914.
(172) Rouleau, A.; Ligneau, X.; Tardivel-Lacombe, J.; Morisset, S.; Gbahou, F.; Schwartz, J.-C.; Arrang, J.-M. Histamine H_3-receptor-mediated [^{35}S]GTPγ[S] Binding: Evidence for

Constitutive Activity of the Recombinant and Native Rat and Human H_3 Receptors. *Br. J. Pharmacol.* **2002**, *135*, 383-392.

(173) Schwartz, J.-C.; Morisset, S.; Rouleau, A.; Ligneau, X.; Gbahou, F.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Arrang, J. M. Therapeutic Implications of Constitutive Activity of Receptors: The Example of the Histamine H_3 Receptor. *J. Neural Transm. Suppl.* **2003**, *64*, 1-16.

(174) Krause, M.; Stark, H.; Schunack, W. Medicinal Chemistry of Histamine H_3 Receptor Agonists. *The Histamine H_3 Receptor, A Target for New Drugs.*; Elsevier: Amsterdam, 1998.

(175) Onodera, K.; Watanabe, T. Histamine H_3 Antagonists as Potential Therapeutics in the CNS. *The Histamine H_3 Receptor, A Target for New Drugs.*; Elsevier: Amsterdam, 1998.

(176) Miyazaki, S.; Imaizumi, M.; Onodera, K. Effects of Thioperamide, a Histamine H_3 Antagonist, on a Scopolamine-induced Learning Deficit Using an Elevated Plus-Maze Test in Mice. *Life Sci.* **1995**, *57*, 2137-2144.

(177) Onodera, K.; Miyazaki, S.; Imaizumi, M.; Stark, H.; Schunack, W. Improvement by FUB 181, a Novel Histamine H_3-Receptor Antagonist, of Learning and Memory in the Elevated Plus-maze Test in Mice. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *357*, 508-513.

(178) Passani, M. B.; Cangioli, I.; Bacciottini, L.; Mannaioni, P. F. Thioperamide and Cimetidine Modulate Acetylcholine Release from the Amygdala of Freely Moving Rats. *Inflamm. Res.* **2000**, *49*, S43-44.

(179) Yokoyama, H.; Onodera, K.; Iinuma, K.; Watanabe, T. Effect of Thioperamide, a Histamine H_3 Receptor Antagonist, on Electrically Induced Convulsions in Mice. *Eur. J. Pharmacol.* **1993**, *234*, 129-133.

(180) Yokoyama, H.; Onodera, K.; Maeyama, K.; Sakurai, E.; Iinuma, K.; Leurs, R.; Timmerman, H.; Watanabe, T. Clobenpropit (VUF 9153), a New Histamine H_3 Receptor Antagonists, Inhibits Electrically Induced Convulsions in Mice. *Eur. J. Pharmacol.* **1994**, *260*, 23-28.

(181) Schlicker, E.; Marr, I. The Moderate Affinity of Clozapine at H_3 Receptors is not shared by its two Major Metabolites and by Structurally Related and Unrelated Atypical Neuroleptics. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1996**, *353*, 290-294.

(182) Pillot, C.; Ortiz, J.; Heron, A.; Ridray, S.; Schwartz, J.-C.; Arrang, J. M. Cyproxifan, a Histamine H_3 Antagonist/Inverse Agonist, potentiates Neurochemical and Behavioral Effects of Haloperidol in the Rat. *J. Neurosci.* **2002**, *22*, 7272-7280.

(183) Sakata, T.; Yoshimatsu, H.; Kurokawa, M. Hypothalamic Neuronal Histamine: Implications of its Homeostatic Control of Energy Metabolism. *Nutrition* **1997**, *13*, 403-411.

(184) Takahashi, K.; Suwa, H.; Ishikawa, T.; Kotani, H. Targeted Disruption of H_3 Receptors Results in Changes in Brain Histamine Tone Leading to an Obese Phenotype. *J. Clin. Invest.* **2002**, *110*, 1791-1799.

(185) Bray, G. A.; Tartaglia, L. A. Medicinal Strategies in the Treatment of Obesity. *Nature* **2000**, *404*, 672-677.

(186) Levi, R.; Smith, N. C. Histamine H_3 Receptors: A New Frontier in Myocardial Ischemia. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 825-830.

(187) McLeod, R. L.; Aslanian, R.; Del Prado, M.; Duffy, R.; Egan, R. W.; Kreutner, W.; McQuade, R.; Hey, J. A. Sch 50971, an Orally Active Histamine H_3 Receptor Agonist, Inhibits Central Neurogenic Vascular Inflammation and Produces Sedation in the Guinea Pig. *J. Pharmacol. Exp. Ther.* **1998**, *287*, 43-50.

(188) Bertaccini, G.; Coruzzi, G.; Poli, E. The Histamine H_3 Receptor: a Novel Prejunctional Receptor Regulating Gastro-intestinal Function. *Aliment Pharmacol. Ther.* **1991**, *5*, 585-591.

(189) Mignot, E.; Taheri, S.; Nishino, S. Emerging Therapeutic Targets for Sleep Disorders. *Nature Neurosci.* **2002**, *5*, 1071-1075.

(190) Leurs, R.; Vollinga, R. C.; Timmerman, H. The Medicinal Chemistry and Therapeutic Potential of Ligands for the Histamine H_3 Receptor. *Prog. Drug. Res.*; Birkhauser Verlag: Basel (Switzerland), 1995; pp 107-165.

(191) Arrang, J.-M.; Roy, J.; Morgat, J. L.; Schunack, W.; Schwartz, J.-C. Histamine H_3 Receptor Binding Sites in Rat Brain Membranes: Modulations by Guanine Nucleotides and Divalent Cations. *Eur. J. Pharmacol.* **1990**, *188*, 219-227.
(192) Howson, W.; Parsons, M. E.; Raval, P.; Swayne, G. T. G. Two Novel, Potent and Selective Histamine H_3 Receptor Agonists. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 77-78.
(193) Van der Goot, H.; Schepers, M. J. P.; Sterk, G. J.; Timmerman, H. Isothiourea Analogues of Histamine as Potent Agonists or Antagonists of the Histamine H_3-Receptor. *Eur. J. Med. Chem.* **1992**, *27*, 511-517.
(194) Vollinga, R. C.; de Koning, J. P.; Jansen, F. P.; Leurs, R.; Menge, W. M. P. B.; Timmerman, H. A New Potent and Selective Histamine H_3 Receptor Agonist, 4-(1*H*-Imidazol-4-ylmethyl)piperidine. *J. Med. Chem.* **1994**, *37*, 332-333.
(195) Hey, J. A.; Del Prado, M.; Egan, R. W.; Kreutner, W.; Chapman, R. W. (R)-α-methylhistamine Augments Neural, Cholinergic Bronchospasm in Guinea-pig by Histamine H_1 Receptor Activation. *Eur. J. Pharmacol.* **1992**, *211*, 421-426.
(196) Coruzzi, G.; Gambarelli, E.; Bertaccini, G.; Timmerman, H. Cardiovascular Effects of Selective Agonists and Antagonists of Histamine H_3 Receptors in the Anaesthetized Rat. *Arch. Pharmacol.* **1995**, *351*, 569-575.
(197) Leurs, R.; Tulp, M. T.; Menge, W. M. B. P.; Adolfs, M. J.; Zuiderveld, O. P.; Timmerman, H. Evaluation of the Receptor Selectivity of the H_3 Receptor Antagonists, Iodophenpropit and Thioperamide: an Interaction with the 5-HT_3 Receptor Revealed. *Br. J. Pharmacol.* **1995**, *116*, 2315-2321.
(198) Kitbunnadaj, R.; Zuiderveld, O. P.; Christophe, B.; Hulscher, S.; Menge, W. M. P. B.; Gelens, E.; Snip, E.; Bakker, R. A.; Celanire, S.; Gillard, M.; Talaga, P.; Timmerman, H.; Leurs, R. Identification of 4-(1*H*-Imidazol-4(5)-ylmethyl)pyridine (Immethridine) as a Novel, Potent, and Highly Selective Histamine H_3 Receptor Agonist. *J. Med. Chem.* **2004**, *47*, 2414-2417.
(199) Govoni, M.; Lim, H. D.; El-Atmioui, D.; Menge, W. M. P. B.; Bakker, R. A.; Timmerman, H.; Leurs, R. A Chemical Switch for the Modulation of the Functional Activity of Higher Homologs of Histamine on the Human Histamine H_3 Receptor: Effect of Various Substitutions at the Primary Amino Function. **to be published**.
(200) Leurs, R. How to Bind and Activate Histamine H_1 Receptors?; XXXIII Annual Meeting of the European Histamine Research Society: Düsseldorf/Köln, Germany, 2004.
(201) Stark, H. Recent Advances in Histamine H_3/H_4 Receptor Ligands. *Expert Opin. Ther. Pat.* **2003**, *13*, 851-865.
(202) Sasse, A.; Stark, H.; Reidemeister, S.; Huls, A.; Elz, S.; Ligneau, X.; Ganellin, C. R.; Schwartz, J. C.; Schunack, W. Novel Partial Agonists for the Histamine H_3 Receptor with High in Vitro and in Vivo Activity. *J. Med. Chem.* **1999**, *42*, 4269-4274.
(203) Sasse, A.; Ligneau, X.; Rouleau, A.; Elz, S.; Ganellin, C. R.; Arrang, J. M.; Schwartz, J. C.; Schunack, W.; Stark, H. Influence of Bulky Substituents on Histamine H_3 Receptor Agonist/Antagonist Properties. *J. Med. Chem.* **2002**, *45*, 4000-4010.
(204) Pelloux-Léon, N.; Fkyerat, A.; Piripitsi, A.; Tertiuk, W.; Schunack, W.; Stark, H.; Garbarg, M.; Ligneau, X.; Arrang, J.-M.; Schwartz, J.-C.; Ganellin, C. R. Meta-Substitued Aryl(thio)ethers as Potent Partial Agonists (or Antagonists) for the Histamine H3 Receptor Lacking a Nitrogen Atom in the Side Chain. *J. Med. Chem.* **2004**, *47*, 3264-3274.
(205) Phillips, J. G.; Ali, S. M. Medicinal Chemistry of Histamine H_3 Receptor Antagonists. *The Histamine H_3 Receptor: A Target for New Drugs.*; Elsevier: Amsterdam, 1998; pp 197-222.
(206) Jansen, F. P.; Wu, T. S.; Voss, H. P.; Steinbusch, H. W. M.; Vollinga, R. C.; Rademaker, B.; Bast, A.; Timmerman, H. Characterization of the Binding of the First Selective Radiolabeled Histamine H_3-receptor Antagonist, ^{125}I-iodophenpropit, to Rat Brain. *Br. J. Pharmacol.* **1994**, *113*, 355-362.
(207) Hüls, A.; Purand, K.; Stark, H.; Reidemeister, S.; Ligneau, X.; Arrang, J. M.; Schwartz, J. C.; Schunack, W. Novel Histamine H_3-Receptor Antagonists with Benzyl Ether Structure or Related Moieties: Synthesis and Structure-Activity Relationships. *Arch. Pharm. Pharm. Med. Chem.* **1996**, *329*, 379-385.

(208) Ligneau, X.; Lin, J.-S.; Vanni-Mercier, G.; Jouvet, M.; Muir, J. L.; Ganellin, C. R.; Stark, H.; Elz, S.; Schunack, W.; Schwartz, J.-C. Neurochemical and Behavioural Effects of Ciproxyfan, a Potent Histamine H3-receptor Antagonist. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 658-666.

(209) Liu, H.; Kerdesky, F. A.; Black, L. A.; Fitzgerald, M.; Henry, R.; Esbenshade, T. A.; Hancock, A. A.; Bennani, Y. L. An Efficient Multigram Synthesis of the Potent Histamine H_3 Antagonist GT-2331 and the Reassessment of the Absolute Configuration. *J. Org. Chem.* **2004**, *69*, 192-194.

(210) Tedford, C. E.; Phillips, J. G.; Gregory, R.; Pawlowski, G. P.; Fadnis, L.; Khan, A.; Ali, S. M.; Handley, M. K.; Yates, S. L. Development of *trans*-2-[1*H*-Imidazol-4-yl] Cyclopropane Derivatives as New High-Affinity Histamine H_3 Receptor Ligands. *J. Pharm. Exp. Ther.* **1999**, *289*.

(211) Fox, G. B.; Bao Pan, J.; Esbenshade, T. A.; Bennani, Y. L.; Black, L. A.; Faghih, R.; Hancock, A. A.; Decker, M. W. Effects of Histamine H_3 Receptor Ligands GT-2331 and Cyproxifan in a Repeated Acquisition Avoidance Response in the Spontaneously Hypertensive Rat Pup. *Behav. Brain Res.* **2002**, *131*, 151-161.

(212) Gbahou, F.; Rouleau, A.; Morisset, S.; Parmentier, R.; Crochet, S.; Lin, J.-S.; Ligneau, X.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Schwartz, J.-C.; Arrang, J.-M. Protean Agonism at Histamine H_3 Receptors *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci.* **2003**, *100*, 11086-11091.

(213) Kitbunnadaj, R.; Zuiderveld, O. P.; De Esch, I. J. P.; Vollinga, R. C.; Bakker, R. A.; Lutz, M.; Spek, A. L.; Cavoy, E.; Deltent, M.-F.; Menge, W. M. P. B.; Timmerman, H.; Leurs, R. Synthesis and Structure-Activity Relationships of Conformationally Constrained Histamine H_3 Receptor Agonists. *J. Med. Chem.* **2003**, *46*, 5445-5457.

(214) Liu, C.; Wilson, S. J.; Kuei, C.; Lovenberg, T. W. Comparison of Human, Mouse Rat, and Guinea Pig Histamine H_4 Receptors Reveals Substantial Pharmacological Species Variation. *J. Pharm. Exp. Ther.* **2001**, *299*, 121-130.

(215) Oda, T.; Matsumoto, S. I.; Masuho, Y.; Takasaki, J.; Matsumoto, M.; Kamohara, M.; Saito, T.; Ohishi, T.; Soga, T.; Hiyama, H.; Matsushime, H.; Furuichi, K. cDNA Cloning and Characterization of Porcine Histamine H_4 Receptor. *Biochem. Biophys. Acta* **2002**, *1575*, 135-138.

(216) Gantner, F.; Sakai, K.; Tusche, M. W.; Cruikshank, W. W.; Center, D. M.; Bacon, K. B. Histamine H_4 and H_2 Receptors Control Histamine-Induced Interleukin-16 Release from Human $CD8^+$ T Cells. *J. Pharm. Exp. Ther.* **2002**, *303*, 300-307.

(217) Lim, H. D.; Bakker, R. A.; De Esch, I. J. P.; Leurs, R. Activity Modulation of the Human Histamine H_4 Receptor by Hydrophobic Group-containing Ligands.; XXXIII Annual Meeting of the European Histamine Research Society: Düsseldorf/Köln, Germany, 2004.

(218) Hashimoto, T.; Harusawa, S.; Araki, L.; Zuiderveld, O. P.; Smit, M. J.; Imazi, T.; Takashima, S.; Yamamoto, Y.; Sakamoto, Y.; Kurihara, T.; Leurs, R.; Bakker, R. A.; Yamatodani, A. A Selective Human H_4-Receptor Agonist: (-)-2-Cyano-1-methyl-3-{(2*R*,5*R*)-5-[1*H*-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine. *J. Med. Chem.* **2003**, *46*, 3162-3165.

(219) Jablonowski, J. A.; Grice, C. A.; Chai, W.; Dvorak, C. A.; Venable, J. D.; Kwok, A. K.; Ly, K. S.; Wei, J.; Baker, S. M.; Desai, P. J.; Jiang, W.; Wilson, S. J.; Thurmond, R. L.; Karlsson, L.; Edwards, J. P.; Lovenberg, T. W.; Carruthers, N. I. The First Potent and Selective Non-Imidazole Human Histamine H_4 Receptor Antagonists. *J. Med. Chem.* **2003**, *46*, 3957-3960.

(220) Thurmond, R. L.; Desai, P. J.; Dunford, P. J.; Fung-Leung, W.-P.; Hofstra, C. L.; Jiang, W.; Nguyen, S.; Riley, J. P.; Sun, S.; Williams, K. N.; Edwards, J. P.; Karlsson, L. A Potent and Selective Histamine H_4 Receptor Antagonist with Anti-Inflammatory Properties. *J. Pharm. Exp. Ther.* **2004**, *309*, 404-413.

(221) Terzioglu, N.; Van Rijn, R. M.; Bakker, R. A.; De Esch, I. J. P.; Leurs, R. Synthesis and Structure-activity Relationships of Indole and Benzimidazole Piperazines as Histamine H_4 Receptor Antagonists. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5251-5256.

(222) Govoni, M.; Lim, H. D.; De Esch, I. J. P.; Bakker, R. A.; Timmerman, H.; Leurs, R. Novel Leads for the Development of Ligands Selectively Targeting the Human Histamine

H_4 Receptor: (*E*)-4-(5-Methyl-1*H*-imidazol-4-yl)-but-3-enylamine and 4-(5-Methyl-1*H*-imidazol-4-yl)-butylamine. **to be published**.

(223) Van Rijn, R. M.; Zuiderveld, O. P.; Kitbunnadaj, R.; Bakker, R. A.; Leurs, R. [^{125}I]Iodophenpropit as an Iodinated Histamine H_4-receptor Radioligand.; XXXII Annual Meeting of the European Histamine Research Society: Noordwijkerhout, The Netherlands, 2003.

(224) Mc Grath, J. C.; Arribas, S.; Daly, C. J. Fluorescent Ligands for the Study of Receptors. *Trends Pharm. Sci.* **1996**, *17*, 393-399.

(225) Harris, D. A.; Bashford, C. L. *Spectrophotometry and Spectrofluorimetry: A Practical Approach.*; IRL Press: Oxford, 1987.

(226) Baker, J. G.; Hall, I. P.; Hill, S. J. Pharmacology and Direct Visualization of BODIPY TMR-CGP: a Long Acting Fluorescent β_2-Adrenoceptor Antagonists. *Br. J. Pharmacol.* **2003**, *139*, 232-242.

(227) Briddon, S. J.; Middleton, R. J.; Cordeaux, Y.; Flavin, F. M.; Weinstein, J. A.; George, M. W.; Kellam, B.; Hill, S. J. Quantitative Analysis of the Formation and Diffusion of A_1-Adenosine Receptor-Antagonist Complexes in Single Living Cells. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4673-4678.

(228) Briddon, S. J.; Middleton, R. J.; Yates, A. S.; George, M. W.; Kellam, B.; Hill, S. J. Application of Fluorescence Correlation Spectroscopy to the Measurement of Agonist Binding to a G-Protein Coupled Receptor at the Single Cell Level. *Faraday Discuss.* **2004**, *126*, 197-207.

(229) Minsky, M. Microscopy Apparatus: US 3013467, 1961.

(230) Inoue, S. Foundations of Confocal Scanned Imaging in Light Microscopy. *Handbook of Biological Confocal Microscopy*; Plenum Press: New York, 1995; pp 1-17.

(231) Fine, A. Confocal Microscopy: Principle and Practice. *Imaging Neurons: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: New York, 1999; pp 11.11-11.11.

(232) Baindur, N.; Triggle, D. J. Selective Fluorescent Ligands for Pharmacological Receptors. *Drug Dev. Res.* **1994**, *33*, 373-398.

(233) Baindur, N.; Triggle, D. J. Concept and Progress in the Development and Utilization of Receptor-specific Fluorescent Ligands. *Med. Res. Rev.* **1994**, *14*, 591-664.

(234) Tairi, A. P.; Hovius, R.; Pick, H.; Blasey, H.; Bernard, A.; Surprenant, A.; Lunström, K.; Vogel, H. Ligand Binding to the Serotonin 5-HT_3 Receptor Studied With a Novel Fluorescent Ligand. *Biochemistry* **1998**, *37*, 15850-15864.

(235) Madsen, B. W.; Beglan, C. L.; Spivak, C. E. Fluorescein-labeled Naloxone Binding to m Opioid Receptors on Live Chinese Hamster Ovary Cells Using Confocal Fluorescent Microscopy. *J. Neurosci. Methods* **2000**, *97*, 123-131.

(236) Beaudet, A.; Nouel, D.; Stroh, T.; Vandenbulcke, F.; Dal-Farra, C.; Vincent, J. P. Fluorescent Ligands for Studying Neuropeptide Receptors by Confocal Microscopy. *Braz. J. Med. Biol. Res.* **1998**, *31*, 1479-1489.

(237) Terillon, S.; Cheng, L. L.; Stoev, S.; Mouillac, B.; Barberis, C.; Manning, M.; Durroux, T. Synthesis and Characterization of Fluorescent Antagonists and Agonists for Human Oxytocin and Vasopressin V1a Receptors. *J. Med. Chem.* **2002**, *45*, 2579-2588.

(238) Valloton, P.; Tairi, A. P.; Wohland, T.; Friedrich-Benet, K.; Pick, H.; Hovius, R.; Vogel, H. Mapping the Antagonist Binding Site of the Serotonin Type 3 Receptor by Fluorescence Resonance Energy Transfer. *Biochemistry* **2001**, *40*, 12237-12242.

(239) Turcatti, G.; Nemeth, K.; Edgerton, M. D.; Knowles, J.; Vogel, H.; Chollet, A. Fluorescent Labeling of NK2 Receptor at Specific Sites in vivo and Fluorescence Energy Transfer Analysis of NK2 Ligand-receptor Complexes. *Receptor Channels* **1997**, *5*, 201-207.

(240) Turcatti, G.; Zoffmann, S.; Lowe, J. A.; Drozda, S. E.; Chassaing, G.; Schwartz, T. W.; Chollet, A. Characterization of Non-peptide Antagonist and Peptide Agonist Binding Sites of the NK1 Receptor with Fluorescent Ligands. *J. Biol. Chem.* **1997**, *272*, 21167-21175.

(241) Turcatti, G.; Nemeth, K.; Edgerton, M. D.; Meseth, U.; Talabot, F.; Peitsch, M.; Knowles, J.; Vogel, H.; Chollet, A. Probing the Structure and Function of the Tachykinin Neurokinin-2 Receptor Through Biosynthetic Incorporation of Fluorescent Amino Acids at Specific Sites. *J. Biol. Chem.* **1996**, *271*, 19991-19998.

(242) Brahmadevara, N.; MacDonald, A.; McGrath, J. C.; Daly, C. J. Distribution of β-adrenoceptors in the Wall of Rat Aorta. *Br. J. Pharmacol.* **2002**, *137*, 93P.
(243) Macchia, M.; Salvetti, F.; Bertini, S.; Di Bussolo, V.; Gattuso, L.; Gesi, M.; Hamdan, M.; Klotz, K. N.; Laragione, T.; Lucacchini, A.; Minutolo, F.; Nencetti, S.; Papi, C.; Tuscano, D.; Martini, C. 7-Nitrobenzofurazan (NBD) Derivatives of 5'-*N*-Ethylcarboxamidoadenosine (NECA) as New Fluorescent Probes for Human A_3 Adenosine Receptors. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3023-3026.
(244) Daly, C. J.; Milligan, C. M.; Milligan, G.; Mackenzie, J. F.; McGrath, J. C. Cellular Localization and Pharmacological Characterization of Functioning *Alpha*-1 Adrenoceptors by Fluorescent Ligand Binding and Image Analysis Reveals Identical Binding Properties of Clustered and Diffuse Populations of Receptors. *J. Exp. Pharm. Ther.* **1998**, *286*, 984-990.
(245) Berque-Bestel, I.; Soulier, J. L.; Giner, M.; Rivail, L.; Langlois, M.; Sicsic, S. Synthesis and Characterization of the First Fluorescent Antagonists for Human 5-HT_4 Receptors. *J. Med. Chem.* **2003**, *46*, 2606-2620.
(246) Harikumar, H. G.; Pinon, D. I.; Wessels, W. S.; Prendergast, F. G.; Miller, L. J. Environment and Mobility of a Series of Fluorescent Reporters at the Amino Terminus of Structurally Related Peptide Agonist and Antagonists bound to the Cholecystokinin Receptor. *J. Biol. Chem.* **2002**, *277*, 18552-18560.
(247) Pramanik, A.; Rigler, R. Ligand-receptor Interactions in the Membrane of Cultured Cells Monitored by Fluorescence Correlation Spectroscopy. *Biol. Chem.* **2001**, *382*, 371-378.
(248) Zhong, Z.-H.; Pramanik, A.; Ekberg, K.; Jansson, O. T.; Jörnvall, H.; Wahren, J.; Rigler, R. Insulin Binding Monitored by Fluorescence Correlation Spectroscopy. *Diabetologia* **2001**, *44*, 1184-1188.
(249) Patel, R. C.; Kumar, U.; Lamb, D. C.; Eid, J. S.; Rocheville, M.; Grant, M.; Rani, A.; Hazlett, T.; Patel, S. S.; Gratton, E.; Patel, Y. C. Ligand Binding to Somatostatin Receptors Induces Receptor-specific Oligomer Formation in Live Cells. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 3294-3299.
(250) Rigler, R.; Pramanik, A.; Jonasson, P.; Kratz, G.; Jansson, O. T.; Nygren, P.-Å.; Ståhl, S.; Ekberg, K.; Johansson, B.-L.; Uhlén, S.; Uhlén, M.; Jörnvall, H.; Wahren, J. Specific Binding of Proinsulin C-peptide to Human Cell Membranes. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 13318-13323.
(251) Pick, H.; Preuss, A. P.; Mayer, M.; Wohland, T.; Hovius, R.; Vogel, H. Monitoring Expression and Clustering of the Ionotropic $5HT_3$ Receptor in Plasma Membranes of Live Biological Cells. *Biochemistry* **2003**, *42*, 877-884.
(252) Overton, M. C.; Blumer, K. J. Use of Fluorescence Resonance Energy Transfer to Analyze Oligomerization of G-protein-coupled Receptors Expressed in Yeast. *Methods* **2002**, *27*, 324-332.
(253) Rios, C. D.; Jordan, B. A.; Gomes, I.; Devi, L. A. G-Protein-Coupled Receptor Dimerization: Modulation of Receptor Function. *Pharmacology and Therapeutics* **2001**, *92*, 71-87.
(254) Svoboda, K.; Helmchen, F.; Denk, W.; Tank, D. W. Spread of Dendritic Excitation in Layer 2/3 Pyramidal Neurons in Rat Barrel Cortex *In Vivo*. *Nature Neurosci.* **1999**, *2*, 65-73.
(255) Petty, H. R.; Francis, J. W. Polymorphonuclear Leucocyte Histamine Receptors: Occurrence in Cell Surface Clusters and their Redistribution During Locomotion. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 4332-4335.
(256) Li, L.; Kracht, J.; Peng, S.; Bernhardt, G.; Buschauer, A. Synthesis and Pharmacological Activity of Fluorescent Histamine H_1 Receptor Antagonists Related to Mepyramine. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1245-1248.
(257) Li, L.; Kracht, J.; Peng, S.; Bernhardt, G.; Elz, S.; Buschauer, A. Synthesis and Pharmacological Activity of Fluorescent Histamine H_2 Receptor Antagonists Related to Potentidine. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1717-1720.
(258) Malan, S. F.; Van Marle, A.; Menge, W. M. B. P.; Zuliani, V.; Hoffmann, M.; Timmerman, H.; Leurs, R. Fluorescent Ligands for the Histamine H_2 Receptor: Synthesis and Preliminary Characterization. *Bioorg. Med. Chem.* **2004**, *12*, 6495-6503.

(259) Haugland, R. P. *Handbook of Fluorescent Probes and Reasearch Products.*; Ninth ed.; Molecular Probes, Inc.: Eugene, 2002.
(260) Marinissen, M. J.; Gutkind, J. S. G-Protein-coupled Receptors and Signaling Networks: Emerging Paradigms. *Trends Pharmacol. Sci.* **2001**, *22*, 368-376.
(261) Weiss, J. M.; Morgan, P. H.; Lutz, M. W.; Kenakin, T. P. The Cubic Ternary Complex Receptor-Occupancy Model. III. Resurrecting Efficacy. *J. Theor. Biol.* **1996**, *181*, 381-397.
(262) Kenakin, T. Efficacy at G-protein-coupled Receptors. *Nat. Rev. Drug Discov.* **2002**, *1*, 103-110.

Chapter 2

Scope of this thesis

The biogenic monoamine histamine is nowadays recognised as a chemical messenger implicated in various physiological and pathophysiological conditions. As described in chapter 1, four human G-protein coupled histamine receptors subtypes (H_1R, H_2R, H_3R and H_4R) are currently acknowledged to mediate the diverse actions of histamine. Specific activation or blockade of the four histamine receptor subtypes led to a tremendous increase in the knowledge of the role of histamine in both physiology and pathology.

In 2000, at the start of the studies described in this Ph.D. thesis, it was increasingly documented that G-protein coupled receptors (GPCRs) coupled to signal transduction pathways in the absence of an agonist, resulting in constitutive receptor activity,[1] which consequently led to the pharmacological reclassification of GPCR antagonists into inverse agonists and neutral antagonists. The agonist-independent basal signalling of the H_2 subtype was first observed in 1996 and a neutral antagonist was concomitantly identified.[2] Constitutive signalling of the H_1,[3] H_3[4] and H_4[5] subtypes was detected soon after the start of the research reported in this thesis, but the design of novel histamine ligands, especially for the H_3 and H_4 receptors, was hampered as there was no comprehensive understanding of the molecular features of histamine ligands responsible for affinity and, more importantly, for intrinsic activity.

Aim of the research described in the following chapters was therefore to synthesise and pharmacologically evaluate selected ligands for the histamine H_1, H_3 and H_4 receptors, in order to obtain a better understanding of the structural requirements necessary for agonism, inverse agonism and neutral antagonism on the aforementioned receptors. The new ligands should allow us to draw structure-activity relationships (SARs) for agonists, inverse agonists and neutral antagonists at the histamine H_1, H_3 and H_4 receptors, ultimately leading to a better understanding of the ligand-receptor interactions and therefore to new insights and ideas for the generation of novel classes of histamine ligands.

Chapter 3 describes the synthesis and pharmacological identification of neutral antagonists for the histamine H_1 receptor (H_1R). The identified neutral antagonists exhibit hybrid structures composed of the endogenous agonist histamine combined with the classical H_1R antagonist pharmacophore. Such compounds might be useful pharmacological tools and they may as well serve as starting point for the development of high-affinity neutral H_1R antagonists.

In chapter 4 the synthesis and pharmacological evaluation of imbutamine, impentamine and imhexamine derivatives on the histamine H_3 receptor (H_3R) are reported. The afforded series of compounds allowed the identification of the primary amino function of the abovementioned

higher homologues of histamine as a chemical switch for the modulation of the functional activity of the compounds on the H_3R.

Chapter 5 reports the synthesis of a series of histamine derivatives bearing a longer and/or unsaturated side chain as well as the corresponding 5-methyl substituted analogues. The synthesised compounds were pharmacologically characterized on the H_3R and H_4R, which led to the identification of (*E*)-4-(5-methyl-1*H*-imidazol-4-yl)-but-3-enylamine and 4-(5-methyl-1*H*-imidazol-4-yl)-butylamine as novel leads for the development of ligands selectively targeting the human H_4R.

Chapter 6 deals with the synthesis of targeted imidazole libraries via a solid phase synthesis (SPS) approach. Efforts towards a SPS route to the development of imbutamine and impentamine derivatives as well as the application of a palladium catalysed cross coupling reaction on a solid support for the generation of a library of conformationally constrained analogues of histamine have been reported.

In chapter 7 attempts toward the synthesis of fluorescent conjugates for the H_3R are illustrated. Imbutamine and impentamine were derived with several fluorescent moieties. Some of the synthesised compounds were subsequently evaluated for their H_3R binding affinities and fluorescence properties as well as for their use as H_3R labelling tools in confocal laser-scanning microscopy experiments.

The newly developed glassware and other equipment used for the solid phase synthesis of the imidazole containing compounds (reported in chapter 6) is described in the appendix.

References

(1) Leurs, R.; Smit, M. J.; Alewijnse, A. E.; Timmerman, H. Agonist-independent Regulation of Constitutively Active G-protein Coupled Receptors. *Trends Biochem. Sci.* **1998**, *23*, 418-422.

(2) Smit, M. J.; Leurs, R.; Alewijnse, A. E.; Blauw, J.; Van Nieuw Amerongen, G. P.; Van de Vrede, Y.; Roovers, E.; Timmerman, H. Inverse Agonism of Histamine H_2 Antagonists Accounts for Upregulation of Spontaneously Active Histamine H_2 Receptors. *Proc. Natl. Acad. Sci.* **1996**, *93*, 6802-6807.

(3) Bakker, R. A.; Wieland, K.; Timmerman, H.; Leurs, R. Constitutive Activity of the Histamine H_1 Receptor Reveals Inverse Agonism of Histamine H_1 Receptor Antagonists. *Eur. J. Pharmacol.* **2000**, *387*, R5-R7.

(4) Morisset, S.; Rouleau, A.; Ligneau, X.; Gbahou, F.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Schwartz, J. C.; Arrang, J. M. High Constitutive Activity of Native H_3 Receptors Regulates Histamine Neurons in Brain. *Nature* **2000**, *408*, 860-864.

(5) Morse, K. L.; Behan, J.; Laz, T. M.; West, R. E. J.; Greenfeder, S. A.; Anthes, J. C.; Umland, S.; Wan, Y.; Hipkin, R. W.; Gonsiorek, W.; Shin, N.; Gustafson, E. L.; Qiao, X.; Wang, S.; Hedrick, J. A.; Greene, J.; Bayne, M.; Monsma, F. J. J. Cloning and Characterization of a Novel Human Histamine Receptor. *J. Pharm. Exp. Ther.* **2001**, *296*, 1058-1066.

Chapter 3

Synthesis and pharmacological identification of neutral histamine H_1-receptor antagonists

Journal of Medicinal Chemistry, 2003, 46, 5812-5824

Marinella Govoni[a#], Remko A. Bakker[a#], Ineke van de Wetering[a], Martine J. Smit[a], Wiro M. B. P. Menge[a], Henk Timmerman[a], Sigurd Elz[b], Walter Schunack[c], and Rob Leurs[a].

a) Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

[b)] Institut für Pharmazie, Universität Regensburg, 93040 Regensburg, Germany.

[c)] Institut für Pharmazie, Freie Universität Berlin, Königin-Luise-Straße 2-4, 14195 Berlin (Dahlem), Germany.

[#)] These authors have contributed equally.

Abstract

In the present study we searched for neutral antagonists for the human histamine H_1 receptor (H_1R) by screening newly synthesised ligands that are structurally related to H_1R agonists for their affinity using radioligand displacement studies and by assessing their functional activity via performing a NF-κB driven reporter-gene assay that allows for the detection of both agonistic and inverse agonistic responses. Starting from the endogenous agonist for the H_1R, histamine, we synthesised and tested various analogues and ultimately identified several compounds with partial inverse agonistic properties, and identified two neutral H_1 receptor antagonists, namely 2-[2-(4,4-diphenyl-butyl)-1*H*-imidazol-4-yl]-ethylamine (histabudifen, **18d**) (pK_i = 5.8, α = 0.02) and 2-[2-(5,5-diphenyl-pentyl)-1*H*-imidazol-4-yl]-ethylamine (histapendifen, **18e**) (pK_i = 5.9, α = -0.09).

Introduction

Four human G-protein coupled histamine-receptor subtypes (H_{1-4}) are currently recognised to mediate the various actions of the monoamine histamine.[1-10] Specific activation or blockade of these receptor subtypes has led to a tremendous increase in the knowledge of the roles of histamine in both physiology and pathology. Stimulation of the histamine H_1 receptor (H_1R), which is found throughout the body, causes contraction of smooth muscles in, e.g. the airways and intestine.[11] Moreover, histamine plays a role in allergic conditions that have often been treated successfully with H_1R antagonists.[12]

It is increasingly recognised that G protein-coupled receptors (GPCRs) activate their associated signal transduction pathways not only upon agonist activation but also in the absence of agonists, resulting in constitutive receptor activity. Concomitant with the appreciation of constitutive receptor activity, the phenomenon of inverse agonism has found general acceptance as well.[13-18] Many ligands that were previously thought to act as antagonists actually inhibit the constitutive receptor signalling, indicating their inverse agonistic behaviour. A third class of ligands, the neutral antagonists, although interacting with the receptor, fail to modulate receptor activity. Inverse agonists display negative intrinsic activity (α) in between -1 and 0 and are relatively common, whereas neutral antagonists, ligands without intrinsic activity ($\alpha = 0$), are quite rare but known for several GPCRs, including histamine H_2 and H_3 receptors.[19-21] The actual therapeutic importance of constitutive GPCR activity has not been clarified yet, but various human diseases have been ascribed to constitutive receptor activity induced by mutations in genes encoding GPCRs.[22-26] It is obvious that for these genetic disorders inverse agonists are essential for silencing the mutant GPCRs as neutral antagonists would be of no use.

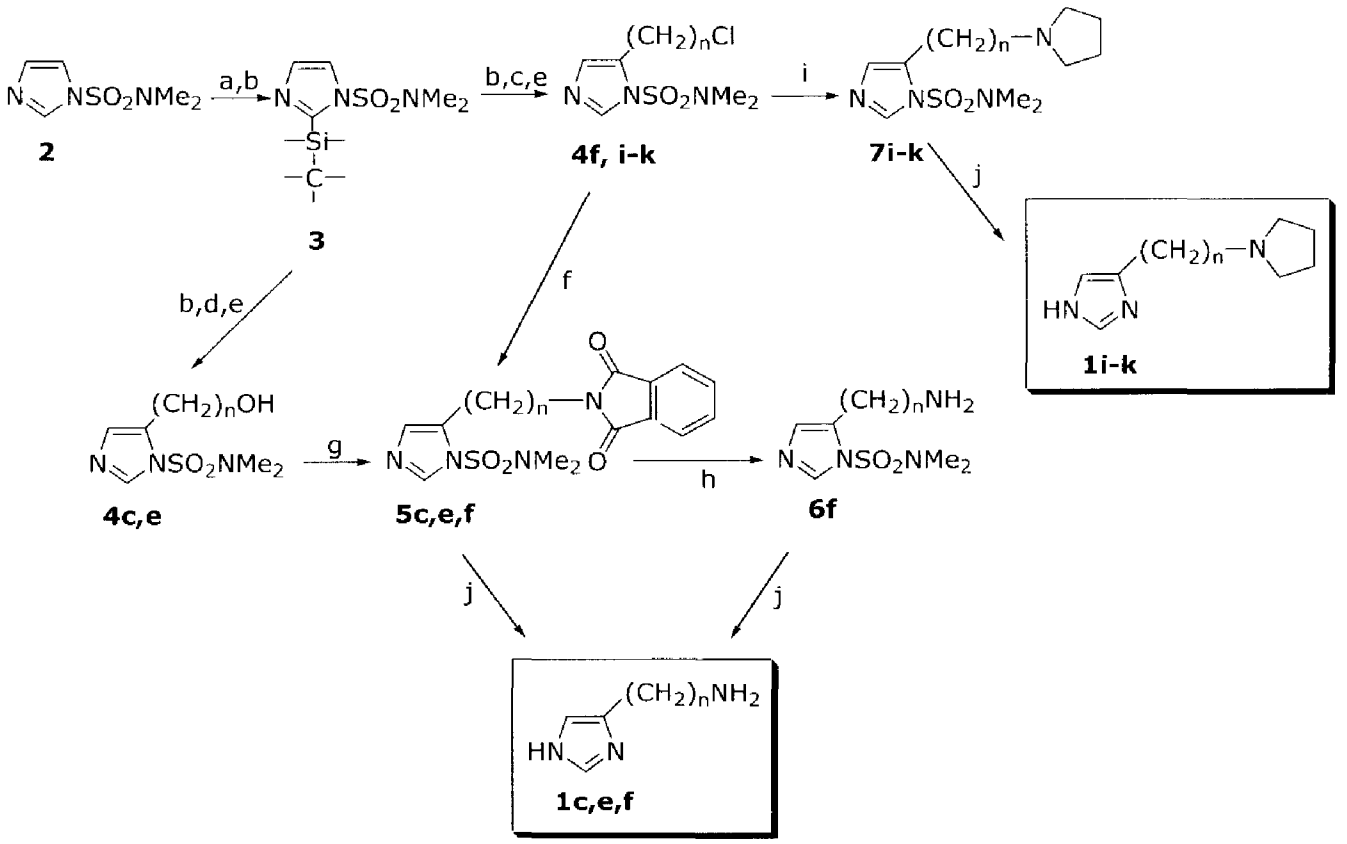
We have recently shown constitutive activity of the wild-type human H_1R and we have identified well-known therapeutics such as cetirizine (Zyrtec®), epinastine (Flurinol®), loratidine (Claritin®) as inverse agonists, which led us to reconsider their mechanism of action.[27,28] No data on the physiological relevance of constitutive H_1R activity are currently available. Constitutive receptor activity can be achieved by high receptor expression-levels, but also by other means, i.e. removal of sodium ions[29] and co-expression of their cognate $G\alpha$ proteins[30-33], or specific $G\beta\gamma$ subunit combinations.[28,34] Moreover, receptor mutation can result in constitutively active (CAM) or inactive (CIM) mutant receptors.[35-38] Therefore, constitutive H_1R activity may contribute to pathophysiological conditions where either H_1R upregulation occurs, such as in patients with allergic rhinitis,[39] or $G\alpha_q$ protein levels are found to be elevated, such as in the guinea pig nasal mucosa in a model of nasal hyper-responsiveness.[40] Although an inverse H_1R agonist would suppress any apparent constitutive H_1R activity, long-term exposure of cells expressing constitutively active GPCRs to inverse agonists may result in receptor up-regulation.[19,41-44] This may have clinical significance since an increased sensitivity,

development of tolerance and recurrence have been attributed to long-term treatment with for instance inverse H_2R agonists.[45,46] However, so far H_1R up-regulation or sensitisation has not been reported upon prolonged inverse agonist treatment.

Clearly, the development of a neutral H_1R antagonist would give a valuable pharmacological tool to study the potential physiological role of constitutive H_1R activity which, to date, has been hampered by the lack of neutral H_1R antagonists. In the present study we report the synthesis of a variety of histamine homologues, their binding affinities and their intrinsic H_1R activity using a reporter-gene assay.

Chemistry

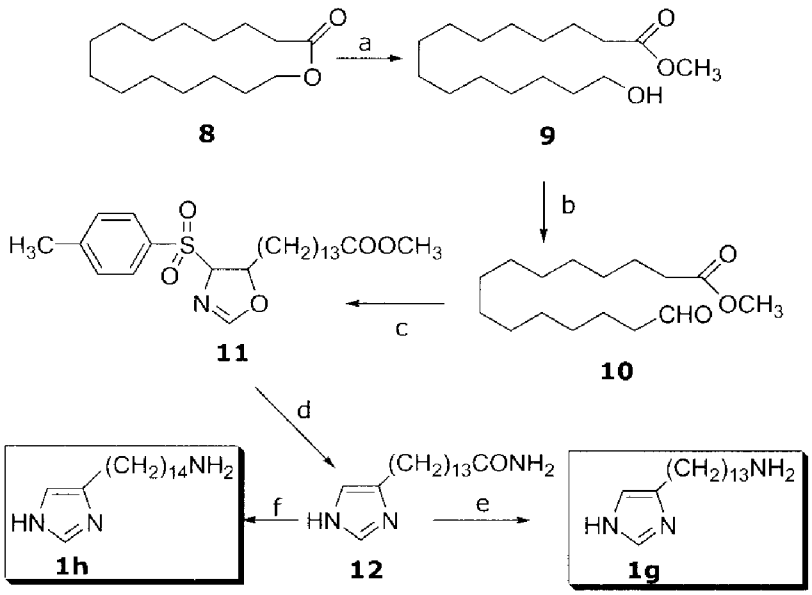
The target aminoalkylimidazoles **1c,e-k** were prepared by lithiation of a suitable 1,2-diprotected imidazole **3**[47] and subsequent treatment with 1-chloro-ω-iodoalkanes or 2-(ω-bromo-alkyloxy)-tetrahydro-pyrans to give compounds **4c,e** and **4f,i,j** (Scheme 1).



Scheme 1. Synthesis of compounds **1c,e,f,i-k**. Reagents: (a) *n*-BuLi, THF, -70°C; (b) *tert*-butyldimethylsilyl chloride; (c) 1-chloro-ω-iodoalkane; (d) 2-(ω-bromo-alkyloxy)-tetrahydro-pyran; (e) HCl, r.t.; (f) isoindole-1,3-dione, Na_2CO_3, DMF, 90°C; (g) triphenylphosphine, isoindole-1,3-dione, diisopropylazodicarboxylate, THF; (h) hydrazine monohydrate, EtOH, reflux; (i) pyrrolidine, r.t; (j) 30% HBr, reflux.

The ω-hydroxy group of **4c,e** was converted into a phthaloyl group via a Mitsunobu reaction[48] followed by hydrolysis of the phthalimide group to give the amines **1c,e**. Alternatively, the ω-chloro group of **4f,i,j** was converted either into a pyrrolidine via reaction with pyrrolidine to give compounds **7i-k** or via a Gabriel synthesis[49] into compound **6f**. Deprotection yielded compounds **1f,i-k**.

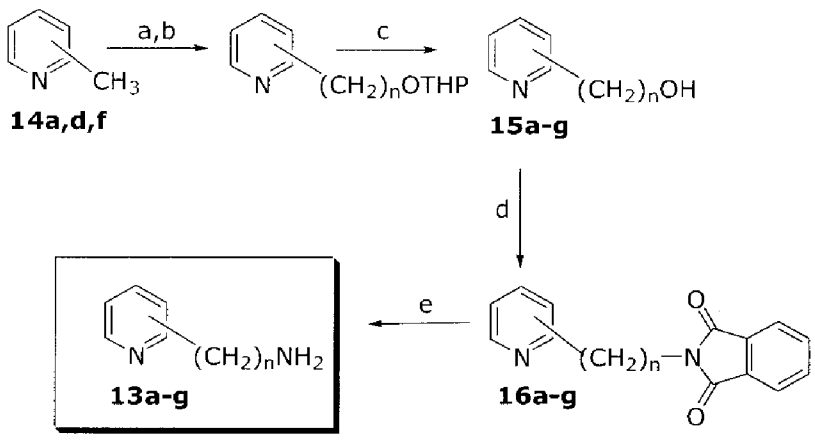
For aminoalkylimidazoles **1g,h** the synthetic pathway shown in Scheme 2 was adopted.



Scheme 2. Synthesis of compounds **1g,h**. Reagents: (a) MeOH, *p*-toluenesulfonic acid monohydrate, reflux; (b) Swern oxidation; (c) TosMIC, NaCN, EtOH abs.; (d) NH_3/MeOH, 90-110°C, 10 atm; (e) PIFA, CH_3CN/H_2O (1/1); (f) $LiAlH_4$, THF.

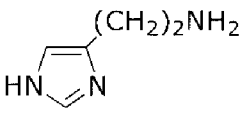
Keystep in this synthesis is the conversion of aldehyde **10** into 4-tosyloxazoline **11** using TosMIC followed by treatment with ammonia in methanol under pressure[50] to give amide **12**. Treatment of amide **12** with [*I,I*-Bis(trifluoroacetoxy)iodo]benzene (PIFA)[51] gave compound **1g**, whereas reduction with $LiAlH_4$ afforded compound **1h**.

The aminoalkylpyridines **13a-g** were prepared according to Scheme 3.

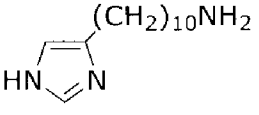


Scheme 3. Synthesis of compounds **13a-g**. Reagents: (a) *n*-BuLi (ortho-isomers) *or* LDA (meta- and para-isomers), THF, -50°C; (b) 2-(ω-bromo-alkyloxy)-tetrahydro-pyran *or* dibromoalkane, -50°C; (c) HCl, r.t.; (d) isoindole-1,3-dione, Na_2CO_3, DMF, 90°C *or* triphenylphosphine, isoindole-1,3-dione, diisopropylazodicarboxylate, THF; (e) hydrazine monohydrate, EtOH, reflux.

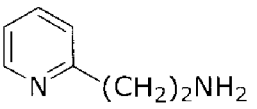
Lithiation of methyl-pyridines **14a,d,f**[52] and coupling with 1,8-dibromooctane or 2-(ω-bromo-alkyloxy)-tetrahydro-pyrans yielded compounds **15a-g**. A Gabriel synthesis or a Mitsunobu reaction gave compounds **16a-g**. The free amino group was obtained by hydrazinolysis resulting in compounds **13a-g**.



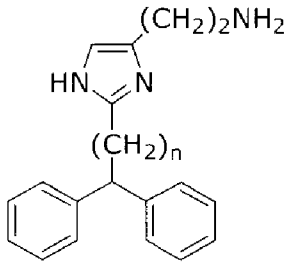
Histamine



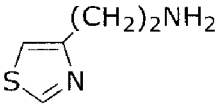
1d;VUF 4734



2-PEA



18a-e;histaprodifens



2-TEA

Figure 1. Structures of 2-[1*H*-imidazol-4-yl]ethylamine (histamine), 2-(thiazol-4-yl) ethanamine (4-TEA), 2-(pyridine-2-yl)ethyl-1-amine (2-PEA), VUF 4734 (**1**), and histaprodifen.

Pharmacological results and discussion

The compound 10-(1*H*-imidazol-4-yl)-decylamine (**1d**; VUF 4734, Figure 1) has been reported to exhibit the highest affinity for the guinea pig H_1R among a series of higher histamine analogues,[53] and subsequently proved to be a partial inverse agonist for the human H_1R (see Table 1 and Figure 2).

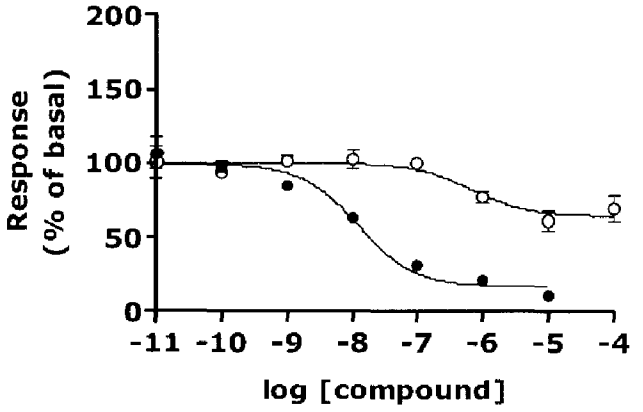
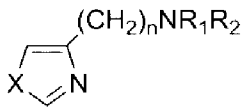


Figure 2. The effects of the full inverse H_1 agonist mepyramine (●) and the partial inverse H_1 agonist VUF 4734 (○) on the basal signalling of the H_1R, as measured by the bioluminescent reporter gene assay (see also Table 1). Data are normalised to the basal signalling observed in the assay (set to 100%).

Therefore, we have prepared a series of ω-(1*H*-imidazol-4-yl)-alkylamines (**1c**, **1e-h**) and investigated the effect of the length of the alkyl spacer of histamine analogues on the human H_1R binding-affinity and intrinsic activity. As shown in Table 1, the H_1R binding affinity for the higher homologues of histamine (**1a-h**) increases with the elongation of the side chain, whereas for the potency a maximum is reached for compound **1e**, bearing an eleven carbon atoms side chain. The intrinsic activity of the compounds decreases as the length of the side chain increases, meaning that the compounds are turning from partial inverse agonists to full inverse agonists.

Table 1. Chemical structures and pharmacological properties of the aminoalkylimidazoles **1a-k** for the human H_1 receptor. The values are expressed as means ± SEM of separate experiments, each performed in triplicate.



compd	name or code	X	n	R_1	R_2	pK_i[a]	pIC_{50}[a]	α[a]
	Histamine	NH	2	H	H	4.2 ± 0.1	6.8 ± 0.1[b]	+ 1.00
	2-TEA	S	2	H	H	< 4	-[c]	-[d]
1a	VUF 4732	NH	6	H	H	3.3 ± 0.4	-[e]	-[e]
1b	VUF 4733	NH	8	H	H	4.4 ± 0.2	5.5 ± 0.8	-0.54 ± 0.13
1c	VUF 5695	NH	9	H	H	4.7 ± 0.2	5.7 ± 0.2	-0.72 ± 0.03
1d	VUF 4734	NH	10	H	H	5.3 ± 0.1	6.3 ± 0.2	-0.54 ± 0.06
1e	VUF 5696	NH	11	H	H	5.6 ± 0.1	6.6 ± 0.1	-0.61 ± 0.04
1f	VUF 5671	NH	12	H	H	6.0 ± 0.1	6.0 ± 0.2	-0.62 ± 0.09
1g	VUF 5697	NH	13	H	H	6.4 ± 0.1	5.9 ± 0.2	-0.84 ± 0.06
1h	VUF 5673	NH	14	H	H	6.6 ± 0.1	5.7 ± 0.1	-0.90 ± 0.11
1i	VUF 5669	NH	8	$-(CH_2)_4-$		5.4 ± 0.1	6.2 ± 0.1	-0.99 ± 0.05
1j	VUF 5670	NH	10	$-(CH_2)_4-$		5.4 ± 0.2	6.4 ± 0.3	-0.61 ± 0.08
1k	VUF 5672	NH	12	$-(CH_2)_4-$		5.7 ± 0.1	5.4 ± 0.1	-0.46 ± 0.33
	Mepyramine					8.7 ± 0.1	7.9 ± 0.1	-1.00

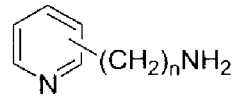
[a] N>3. [b] pEC_{50} value. [c] Could not be estimated. [d] Tested up to 100 μM, no effects up to 10 μM. [e] Low affinity for the receptor, not tested for the efficacy.

In an attempt to obtain compounds with a higher H_1R affinity the amino group of some of the higher homologues of histamine was exchanged for a pyrrolidine moiety (**1i-k**), by analogy with the classical H_1R antagonists for which it is known that the substitution of the primary amino function by a pyrrolidine ring results in an increase in H_1R affinity of an order of magnitude or more.[12] This exchange results in an increased binding affinity of one log unit only in the case of compound **1i**, whereas in the case of longer side chains (**1j** and **1k**) the binding affinities are unchanged or reduced. Moreover, there is no significant difference among the binding affinities of the three compounds bearing a pyrrolidine ring at the end of the side chain (**1i-k**). No definite relation between the length of the side chain and the potency can be observed. Opposite to observations made for the series of compounds bearing a primary amino

function (**1a-h**), in this series the intrinsic activity increases as the length of the side chain increases, meaning that the compounds are turning from full inverse agonists to partial inverse agonists. However, no neutral antagonists were identified in this series.

H_1R specific agonists have been developed by replacement of the imidazole ring for a pyridine ring, which does not influence the capability of the resulting compound to bind to the H_1R, provided that the aminoethyl chain is placed in the 2-position of the pyridine ring,[12,54] as in 2-(pyridine-2-yl)ethanamine (2-PEA, Figure 1). On this basis we decided to investigate the importance of the imidazole ring in the binding affinity and intrinsic H_1R activity of the higher homologues of histamine. Various aminoalkylpyridines have been synthesised in which a long side chain has been attached in the 2- (**13a-c**), 3- (**13d,e**) and 4- (**13f,g**) position of the pyridine ring (Table 2).

Table 2. Chemical structures and pharmacological properties of the aminoalkylpyridines **13a-g** for the human H_1 receptor. The values are expressed as means ± SEM of separate experiments, each performed in triplicate.



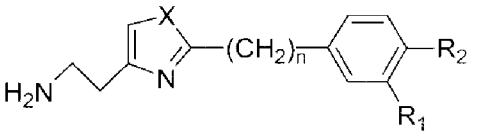
compd	name or code	isomer	n	pK_i[a]	pIC_{50}[a]	α[a]
	2-PEA	ortho	2	3.8 ± 0.1	5.9 ± 0.1[b]	1.11 ± 0.21
13a	VUF 5680	ortho	9	4.5 ± 0.1	5.1 ± 0.2	-0.92 ± 0.08
13b	VUF 5674	ortho	10	4.9 ± 0.1	5.5 ± 0.2[b]	0.40 ± 0.05
13c	VUF 5677	ortho	12	6.0 ± 0.1	-[c]	-[d]
13d	VUF 5675	meta	10	5.1 ± 0.1	5.6 ± 0.1	-0.76 ± 0.01
13e	VUF 5678	meta	12	5.4 ± 0.1	5.9 ± 0.1	-0.78 ± 0.13
13f	VUF 5676	para	10	5.3 ± 0.1	5.6 ± 0.2	-1.00 ± 0.06
13g	VUF 5679	para	12	5.4 ± 0.1	6.4 ± 0.2	-0.67 ± 0.05

[a] N>3. [b] pEC_{50} value. [c] could not be estimated. [d] tested up to 10 μM, no effects up to 1 μM.

For the 2-isomers (**13a-c**) an increase in the H_1R binding-affinity with the elongation of the side chain can be observed, similarly to what happens for the aminoalkylimidazoles with a primary amino function (compounds **1c**, **1d** and **1f**), and the pK_i values are comparable to those of the aminoalkylimidazoles. Although **13b** and especially **13c** appear to be lacking intrinsic H_1R activity, these compounds exhibited non-H_1R mediated effects at high concentrations that interfered with an accurate estimation of their intrinsic H_1R activity in our cell based assay (data not shown). Interestingly, the 3-(**13d,e**) and 4-(**13f,g**) isomers are still active on the receptor, although no significant differences are observed for the binding affinities with elongation of the side chain or shifting the side chain from the meta to the para position. All the aminoalkylpyridines are (partial) inverse H_1R agonists, with the exception of 2-PEA (Table 2), but there is no apparent relation between the length or the position of the side chain and the efficacy of the compound.

Substitution of the 2-position of histamine has proven to be a successful strategy to obtain H_1R-selective agonists[55] such as the full agonist histaprodifen (see Figure 1 and Table 3).[56]

Table 3. Chemical structures and pharmacological properties of compounds **17a-e** and **19a-d** for the human H_1 receptor. The values are expressed as means ± SEM of separate experiments, each performed in triplicate.



compd	name or code	n	X	R_1	R_2	pK_i[a]	pEC_{50}[a]	α[a]
17a	VUF 5963	0	NH	Cl	H	5.2 ± 0.1	5.7 ± 0.1	1.28 ± 0.06
17b		0	NH	CF_3	H	5.4 ± 0.1	6.0 ± 0.1	0.91 ± 0.08
17c	VUF 8811	0	NH	Cl	Cl	4.7 ± 0.1	4.3 ± 0.1	0.50 ± 0.07
17d		4	NH	H	H	< 4	-[b]	-[b]
17e		5	NH	H	H	< 4	-[b]	-[b]
19a		0	S	H	H	4.5 ± 0.1	-[c]	-[d]
19b		0	S	CF_3	H	4.9 ± 0.1	-[c]	-[d]
19c		1	S	H	H	4.2 ± 0.2	-[c]	-[d]
19d		1	S	Cl	H	4.5 ± 0.1	-[c]	-[d]

[a] N>3. [b] Low affinity for the receptor, not tested for the efficacy. [c] Could not be estimated. [d] Tested up to 100 μM, no effects up to 10 μM. [e] pIC_{50} value.

As shown in Table 3 the mono-substituted compounds **17a** and **17b** are agonists with comparable binding affinities and potencies. Interestingly, compound **17a** displays an intrinsic activity (α) that is greater than that of histamine itself (Table 3, Figure 3C). The di-substituted compound (**17c**) is a partial agonist, displaying a reduced binding affinity and potency, suggesting that the accommodation in the ligand-binding pocket of the H_1R[57] is hindered due to steric effects of the chlorine atom in the para-position.

Replacement of the imidazole ring of histamine for a thiazole moiety leads to 2-(thiazol-4-yl)ethanamine (4-TEA,[12] Figure 1), with a very low affinity for the receptor (Table 1). Subsequent substitution of the 2-position of 4-TEA with a (substituted) phenyl ring or a (substituted) phenylmethyl residue was achieved by compounds **19a-d**, all displaying a similar (and low) binding affinity for the receptor.

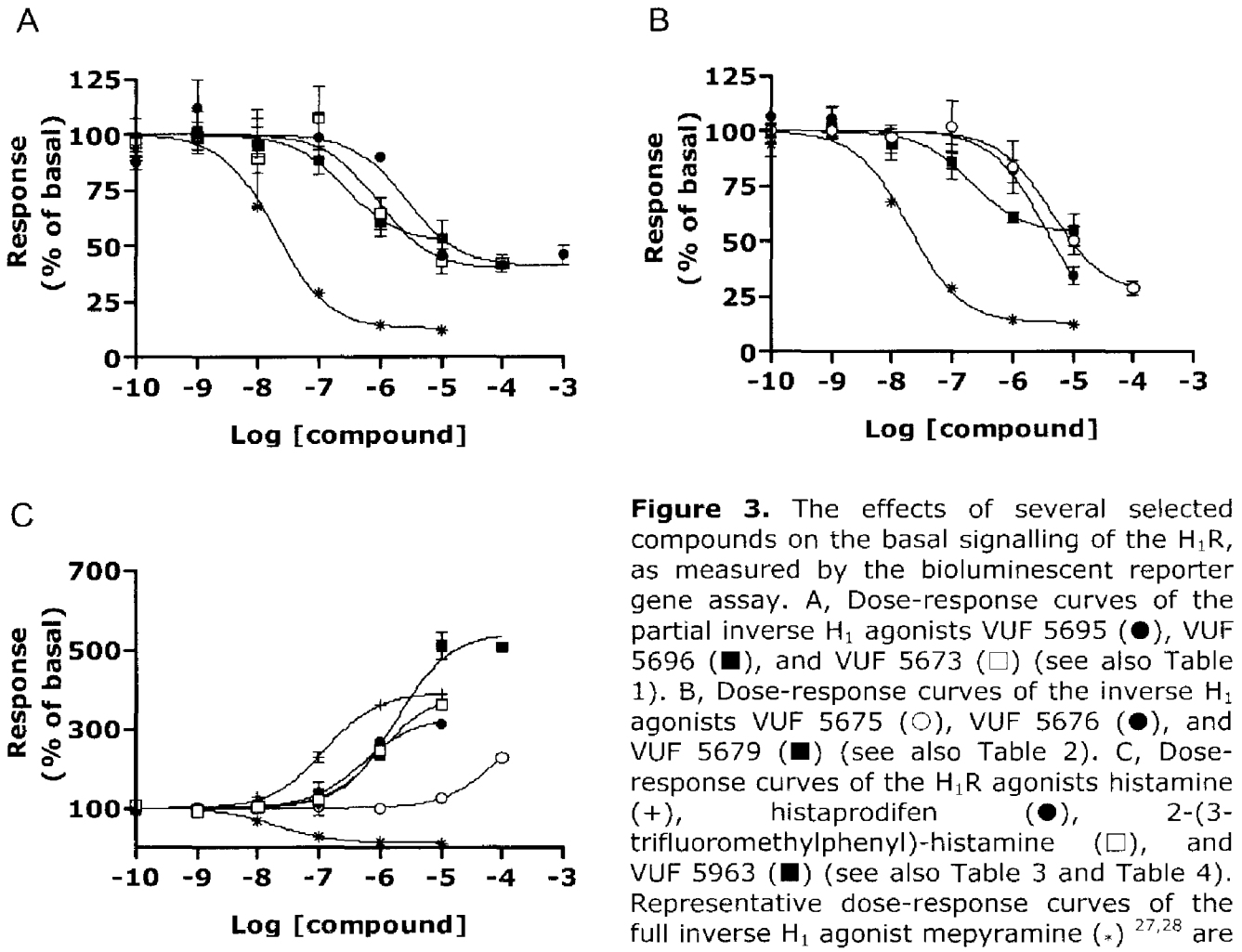
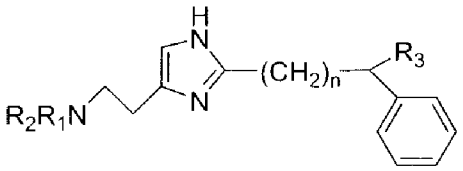


Figure 3. The effects of several selected compounds on the basal signalling of the H_1R, as measured by the bioluminescent reporter gene assay. A, Dose-response curves of the partial inverse H_1 agonists VUF 5695 (●), VUF 5696 (■), and VUF 5673 (□) (see also Table 1). B, Dose-response curves of the inverse H_1 agonists VUF 5675 (○), VUF 5676 (●), and VUF 5679 (■) (see also Table 2). C, Dose-response curves of the H_1R agonists histamine (+), histaprodifen (●), 2-(3-trifluoromethylphenyl)-histamine (□), and VUF 5963 (■) (see also Table 3 and Table 4). Representative dose-response curves of the full inverse H_1 agonist mepyramine (⋆) [27,28] are shown (A, B, and C). Data are normalised to the basal signalling observed in the assay (set to 100%).

Recently, Elz *et al.* synthesised a series of compounds constituting a new class of highly active H_1R agonists that combine a histamine moiety linked at the 2-position with an ω,ω-diphenylalkyl substituent (**18a-e**; histaprodifens, Figure 1), and examined them for H_1-histaminergic properties on the guinea-pig ileum preparation.[58] In this study the affinities, potencies and efficacies of these compounds, and other analogues,[56,58,59] on the cloned human H_1R were determined. The diphenylmethyl substituent present in the histaprodifen-series is a common feature of various clinically used H_1R antagonists,[12] like e.g. cetirizine, and is believed to confer high receptor-affinity on these antihistamines. We investigated the influence of this substituent by testing several substituted histaprodifens and substituted thiazoles.[60,61]

Compounds **18a-i** have interesting structure-activity relationships, as shown in Table 4.

Table 4. Chemical structures and pharmacological properties of compounds **18a-i** and **20a,b** for the human H_1 receptor. The values are expressed as means ± SEM of separate experiments, each performed in triplicate.



compd	n	R_1	R_2	R_3	pK_i	pIC_{50}	α
Histamine		H	H		4.2 ± 0.1	6.8 ± 0.1[b]	+1.00
18a	0	H	H	phenyl	< 4	-[c]	-[c]
18b	1	H	H	phenyl	5.1 ± 0.1	-[d]	-[e]
18c	2	H	H	phenyl	5.7 ± 0.1	6.4 ± 0.1[b]	0.69 ± 0.06
18d	3	H	H	phenyl	5.8 ± 0.1	-[d]	0.02 ± 0.03
18e	4	H	H	phenyl	5.9 ± 0.1	-[d]	-0.09 ± 0.18
18f	2	H	H	cyclohexyl	5.0 ± 0.1	-[d]	-[f]
18g	2	H	H	*p*-Br-phenyl	5.6 ± 0.1	-[d]	-[f]
18h	2	H	H	*p*-CH_3-phenyl	5.5 ± 0.1	-[d]	-[f]
18i	2	-$(CH_2)_4$-		phenyl	5.6 ± 0.1	-[c]	-[d]

[a] N>3. [b] pEC_{50} value. [c] Low affinity for the receptor, not tested for the efficacy. [d] could not be estimated. [e] tested up to 100 μM, no effects up to 10 μM. [f] tested up to 10 μM, no effects up to 1 μM.

The elongation of the spacer results in an increased H_1R binding-affinity (compounds **18b-e**) together with a reduction of the intrinsic activity from full agonism (**18c**) to neutral antagonism (**18d** and **18e**; see also Figure 4).

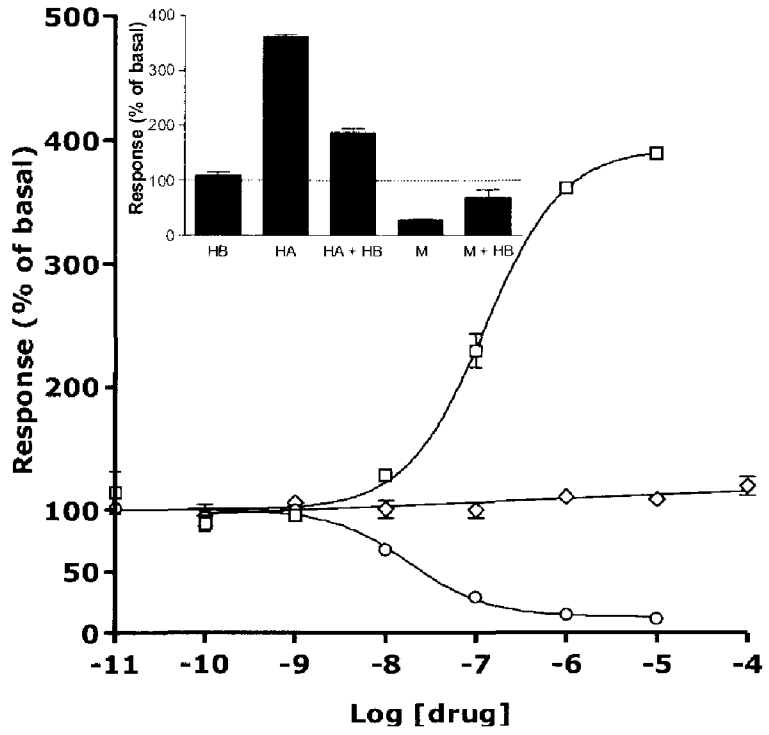


Figure 4. Histabudifen as a neutral H_1R antagonist. The effects of the endogenous agonist histamine (□), the full inverse H_1 agonist mepyramine (○) and histabudifen (◊) on the modulation of basal H_1R signalling, as measured by the bioluminescent reporter gene assay (see also Table 4). Data are normalised to the basal signalling observed in the assay (set to 100%). Inset, Effects of histabudifen (10μM, HB), histamine (1μM, HA), and mepyramine (0.1μM, M) on H_1R-mediated signalling, and the inhibition of histamine- and mepyramine-induced H_1R-mediated responses by histabudifen.

Considering the binding data, the replacement of a phenyl ring with a cyclohexyl ring (**18f**) leads to a reduced affinity for the receptor, and removal of one phenyl ring (**17d,e**) led to a dramatic decrease in binding affinity. However, substitution of one of the phenyl rings in the para-position either with an electron-withdrawing substituent (**18g**) or an electron-releasing substituent (**18h**) does not affect the affinity, suggesting that the electron density on that phenyl ring as well as substitution on the para position are not important for the interaction with the receptor. This striking observation is in contrast with the well-known structure activity relationships for the classical antihistamines[12,62] and with data for agonists.[63] Those studies indicated in fact that para-substitution of the "cis" ring of classical H_1R antagonists with a small lipophilic group (i.e. CH_3, Cl) is favourable and confers the compounds an increased affinity on the receptor. The aromatic character of this ring seems to be indispensable for H_1R activity. In contrast, the second aromatic "trans" ring can be replaced by non-aromatic lipophilic groups (e.g. cyclohexyl) without drastic effects on H_1R-blocking activity.[62] Unlike the known effects for classical H_1R antagonists, the replacement of the primary amino function with a pyrrolidine ring in compound **18i**, does not influence its binding affinity, similarly to what was observed with the series of aminoalkylimidazoles (compounds **1j** and **1k**).

Previously, we have shown the human histamine H_1 receptor to be constitutively active both by measuring the inositolphosphate levels in transiently transfected COS-7 cells, and by measuring the luciferase activity in a reporter-gene assay we set up in which a firefly luciferase reporter-gene is under the transcriptional control of five NF-κB enhancer elements. However, in general, the physiological relevance of inverse agonists needs to be verified in a more physiological environment before the biological effect of antagonists can be primarily ascribed to negative intrinsic activity.[14,15] Therefore, we investigated the effects of (partial) inverse agonists and the identified neutral antagonist for the human histamine H_1-receptors endogenously expressed by HeLa cells.

Long-term exposure of cells expressing constitutively active GPCRs to inverse agonists may result in receptor up-regulation.[19,41-44] Also, sensitisation of receptors by inverse agonist treatment has been shown in cell lines stably expressing receptors at relatively low density[64] as well as in cell lines in which receptor expression is inducible.[65] Although inverse agonist induced receptor up-regulation may have clinical significance,[45,46] no data on the physiological relevance of constitutive H_1R activity are currently available, in part due to the absence of available neutral H_1R antagonists. So far a receptor up-regulation or sensitisation has not been reported for the H_1R upon prolonged inverse agonist treatment. We assessed the long term effects of prolonged inverse agonist treatment of HeLa cells endogenously expressing the H_1R at low expression levels (B_{max} 55 fmol/mg protein, Figure 5A) on the H_1R expression levels and H_1R signalling properties.

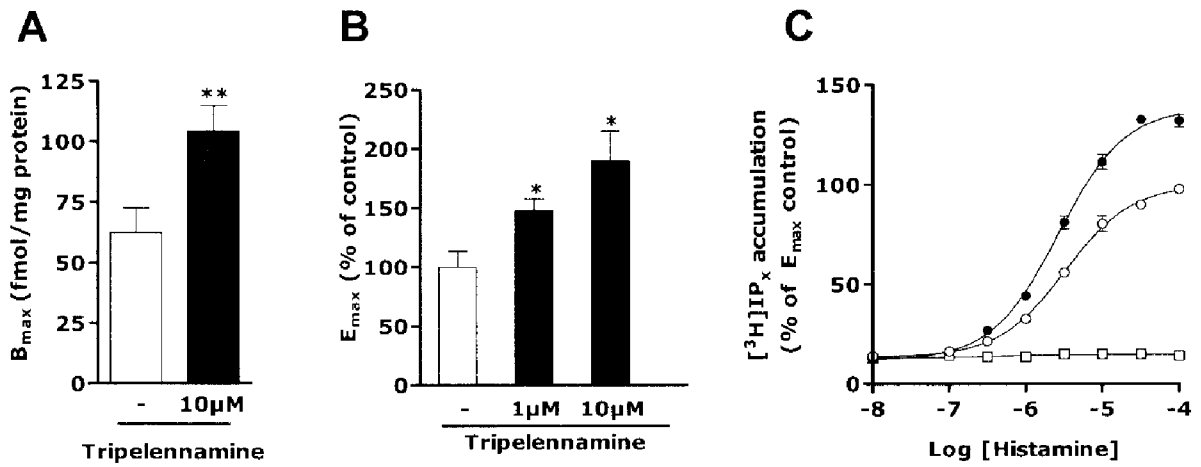


Figure 5. Effects of a 24 h pre-treatment with the inverse H_1 agonist tripelennamine on H_1R expression and H_1R-mediated signalling of HeLa cells endogenously expressing H_1Rs. A, Upregulation of endogenously expressed H_1Rs after a 24h pre-treatment of the cells with 10 µM tripelennamine. B, Increase in the E_{max} of histamine-induced inositolphosphates accumulation upon pre-treatment of the cells with either 1 µM or 10 µM tripelennamine for 24 h. C, Effect of tripelennamine (1 µM, 24h, ●) or histamine (0.63 mM, 24h, □) pre-treatment of HeLa cells on the E_{max} and pEC_{50} of histamine-induced inositolphosphates accumulation in comparison to control cells (○).

The procedure of measuring the inverse-agonist induced up-regulation of H_1Rs is greatly dependent on the dissociation rates of the inverse agonists, as the preparation needs to be free of the inverse agonist used to pretreat the cells, in order to determine either the expression level of the H_1R or its signalling properties. Whereas tripelennamine is one of the few antihistamines that dissociates rather quickly from the receptor and allows for the measurement of H_1R up-regulation and the increase in E_{max} of histamine, many other antihistamines, such as mepyramine and cetirizine, dissociate more slowly from the H_1R.[66] As seen in Figure 5, a 24h treatment of HeLa cells with the inverse H_1R agonist tripelennamine[28] results in a two-fold increase of the total H_1R-expression level (Figure 5A) that is concomitant with a dose-dependent increase in the E_{max}, but not in the EC_{50} value of histamine-induced inositolphosphates formation (Figure 5B and 5C). Although similar effects were observed when mepyramine was used to preincubate the cells, the EC_{50} of histamine after such pre-treatment of the cells was rightward shifted (data not shown), most likely due to the kinetics of mepyramine binding. The maximum enhancement of H_1R signalling observed upon inverse H_1 agonist treatment is a two-fold increase. These data are clearly consistent with the notion of constitutive H_1R activity, and suggests that the inverse H_1R agonist tripelennamine may act to stabilise the H_1R in the cell membrane. In contrast to the effects observed with pretreatment of the cells with the inverse agonist tripelennamine, pretreatment of the cells with 0.63 mM histamine (10 times its K_i value[28] completely prevented histamine stimulated inositolphosphates formation (Figure 5C).

Pre-treatment of HeLa cells with 100μM of the partial inverse H_1R agonist VUF 4734 (**1d**), which corresponds to 20 times its K_i value, did not result in a significant increase of the E_{max} of histamine. The E_{max} of histamine after pre-treatment with VUF 4734 was 146 ± 26 % of that of control cells. Pre-treatment of HeLa cells with 16μM of the identified neutral H_1 antagonist histabudifen (**18d**), corresponding to 10 times its K_i value, did also not result in a significant increase in the E_{max} of histamine compared to control cells. VUF 4734 and histabudifen could not be tested at higher concentrations due to non-H_1R mediated effects in our cell-based assay.

Conclusions

In the present study we reported the synthesis of a variety of histamine homologues and testing of their binding affinities and intrinsic H_1R activities. A NF-κB driven reporter-gene assay allowing testing of H_1R ligands for a dynamic range of intrinsic activities has been used.[28] Moreover, we show an upregulation of endogenously expressed H_1R and an increased effectiveness of histamine to induce the formation of inositolphosphates upon inverse H_1R agonist treatment, underscoring the potential benefit of neutral antagonists.

Novel ligands for the histamine H_1 receptor were synthesised and tested for their affinity and intrinsic activity for the human histamine H_1 receptor and several known H_1-receptor ligands were screened in the same system. Most of the compounds presented in this research are (partial) inverse agonists. Especially aminoalkylimidazoles **1a-k** and aminoalkylpyridines **13a,d-g** are structurally diverse from classical histamine H_1 antagonists, as they possess a structure similar to histamine and H_1 agonists, therefore they constitute a novel class of inverse agonist ligands for this receptor. The substitution of the primary amino function in the aminoalkylimidazole series with a pyrrolidine ring led to the expected increase of affinity for the receptor only in the case of compound **1i**, possibly due to a different binding site for the other ligands compared to classical H_1 antagonists.[62,67] Previously we have indeed proposed different H_1R agonists binding pockets could exist.[57] The position of the nitrogen in the aminoalkylpyridines series does not seem to be important, since all the compounds bind to the receptor. The elongation of the side chain from the classical aminoethyl chain turns the compounds into (partial) inverse agonists, analogous to the aminoalkylimidazoles series, with the exception of compound **13b**, for which we currently have no explanation available.

It has proven difficult to identify neutral H_1-receptor antagonists by modification of the agonist histamine, a strategy previously successfully used to identify neutral H_2- and H_3-receptor antagonists[19,20]. Nonetheless, two histaprodifen-like compounds (**18d** and **18e**), that exhibit hybrid structures composed of the endogenous agonist histamine combined with the classical H_1R antagonist pharmacophore, have been ultimately identified as neutral H_1-receptor antagonists. Consequently, the modification of agonist structures has again proven a

successful strategy to obtain neutral histamine receptor antagonists. The benzhydryl group in compounds **18d** and **18e** seems to be important for their neutral H_1R antagonism. Although at this stage it is not possible to draw definite conclusions on the structural requirements necessary for neutral antagonism on the histamine H_1 receptor, the structure-activity relationships for neutral H_1R antagonists seem to differ from the structure-activity relationships of inverse H_1R agonists. In contrast to the effects of pre-treatment with inverse H_1 agonist, pre-treatment with weak partial inverse agonists or neutral antagonists for the H_1 receptor had no effect on the histamine-induced H_1-receptor mediated responses. These compounds will therefore be useful pharmacological tools and may serve as a starting point for the development of high affinity neutral H_1R antagonists, the availability of which will allow the assessment of the importance of constitutive H_1R receptor activity *in vivo* as well as the requirement of inverse H_1R agonistic properties for the therapeutic value of antihistamines.

Experimental Section

Chemistry

General procedures. Melting points were measured on an Electrothermal IA 9200 apparatus. ^1H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. Chemical shifts are given in ppm downfield from tetramethylsilane as internal standard.
Elemental analyses were performed by the Department of Microanalysis, Groningen University, Groningen, The Netherlands.
Chromatography was performed on J.T.Baker silica gel for flash chromatography.
THF was freshly distilled from $LiAlH_4$ and DMF was dried on molecular sieves.
All reactions were performed under an atmosphere of dry nitrogen.
The 1-chloro-ω-iodoalkanes were prepared by refluxing the corresponding 1,ω-dichloroalkanes with one-third equiv of sodium iodide in acetone and purification by distillation.[68]
The 2-(ω-bromo-alkyloxy)-tetrahydro-pyrans were prepared from the corresponding ω-bromo-alkan-1-ols.[69,70]

5-(9-Hydroxy-nonyl)-imidazole-1-sulfonic acid dimethylamide (4c)
Imidazole-1-sulfonic acid dimethylamide **2**[53] (6.86 g, 0.039 mol) was dissolved in THF (150 mL) and cooled to −70 °C. *n*-Butyllithium in hexane (29.25 mL, 0.047 mol) was added dropwise at −65 °C. After 15 min, a solution of *tert*-butyldimethylsilyl chloride (7.07 g, 0.047 mol) in THF was added at −65 °C and the solution was allowed to warm to room temperature and stirred for an additional 30 min. The mixture was cooled to −70 °C again, and *n*-butyllithium in hexane (24.5 mL, 0.039 mol) was added dropwise. After 30 min, a solution of

2-(9-bromo-nonyloxy)-tetrahydro-pyran (10.00 g, 0.033 mol) in THF was added gradually and the mixture was allowed to (slowly) warm to room temperature (rt) overnight. The reaction mixture was poured into water and the THF was removed under reduced pressure. The product was extracted with dichloromethane, washed with water, dried ($MgSO_4$), and concentrated in vacuo. HCl (37%) was added to the crude product till pH = 1 and stirred for 1 h, then diluted with water, basified with K_2CO_3 and extracted with dichloromethane; the organic layers were combined, dried ($MgSO_4$) and concentrated in vacuo. The product was purified by flash chromatography with ethyl acetate as eluent to afford 1.09 g (10%) of white crystals. ^{1}H NMR ($CDCl_3$): δ 1.20 (m, 10H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 2.60 (t, 2H, imidazole-5-CH_2), 2.78 (s, 6H, $N(CH_3)_2$), 3.53 (t, 2H, CH_2OH), 6.70 (s, 1H, imidazole-4H), 7.78 (s, 1H, imidazole-2H).

5-(11-Hydroxy-undecyl)-imidazole-1-sulfonic acid dimethylamide (4e)

2-(*tert*-Butyl-dimethyl-silanyl)-imidazole-1-sulfonic acid dimethylamide **3**[47] (9.23 g, 0.032 mol) was dissolved in THF (120 mL) and cooled to −70 °C. *n*-Butyllithium in hexane (20 mL, 0.032 mol) was added dropwise at −65 °C. After 30 min, a solution of (11-bromo-undecyloxy)-tetrahydro-pyran (10.63 g, 0.032 mol) in THF was added dropwise and the mixture was allowed to (slowly) warm to room temperature overnight. The solution was acidified with HCl (2 M) and stirred for 1 h, then the solvents were removed in vacuo. The residue was diluted with water, basified with K_2CO_3, extracted with dichloromethane, dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified by flash chromatography switching gradually the eluent from dichloromethane/ethyl acetate (8/2) into ethyl acetate to afford 1.60 g of product (mixed with 25% of **2**) which was used without further purification.

5-(12-Chloro-dodecyl)-imidazole-1-sulfonic acid dimethylamide (4f)

Using the same procedure as for **4c** with 4.46 g (0.026 mol) of **2** and 5.61 g (0.017 mol) of 1-chloro-12-iododododecane gave 2.69 g product (mixed with 45% of 5-(*tert*-butyl-dimethyl-silanyl)-imidazole-1-sulfonic acid dimethylamide) which was used without further purification.

5-(8-Chloro-octyl)-imidazole-1-sulfonic acid dimethylamide (4i)

Using the same approach as for **4e** with 9.23 g (0.032 mol) of **3** and 8.70 g (0.032 mol) of 1-chloro-8-iodooctane gave 9.59 g (93%) of brown oil, which did not need any further purification by flash chromatography. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 8H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.63 (t, 2H, imidazole-5-CH_2), 2.78 (s, 6H, $N(CH_3)_2$), 3.43 (t, 2H, CH_2Cl), 6.73 (s, 1H, imidazole-4H), 7.73 (s, 1H, imidazole-2H).

5-(10-Chloro-decyl)-imidazole-1-sulfonic acid dimethylamide (4j)

Using the same procedure as for **4c** with 7.96 g (0.046 mol) of **2** and 13.05 g (0.043 mol) of 1-chloro-10-iododecane gave 5.32 g product (mixed with 60% of 5-(*tert*-butyl-dimethyl-silanyl)-imidazole-1-sulfonic acid dimethylamide) which was used for the next step without further purification.

5-[9-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-nonyl]-imidazole-1-sulfonic acid dimethylamide (5c)

A mixture of **4c** (1.09 g, 0.0035 mol), triphenylphosphine (0.91 g, 0.0035 mol) and isoindole-1,3-dione (0.51 g, 0.0035 mol) in dry THF (5 mL) was stirred vigorously and placed in an ice/water bath. Diisopropyl azodicarboxylate (0.698 g, 0.00345 mol) in dry THF (5 mL) was added dropwise and stirred at room temperature overnight. The solvent was removed in vacuo and the residue was purified by flash chromatography with ethyl acetate as eluent to afford 0.96 g (63%) of beige solid. ^{1}H NMR ($CDCl_3$): δ 1.28 (m, 10H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.67 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, N(CH_3)$_2$), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 6.78 (s, 1H, imidazole-4H), 7.50-7.78 (m, 5H, isoindole-1,3-dione-H + imidazole-2H).

5-[11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecyl]-imidazole-1-sulfonic acid dimethylamide (5e)

The same procedure as for **5c** starting from 1.60 g (± 0.0046 mol) of **4e** was used. The residue was purified by flash chromatography using ethyl acetate/dichloromethane as eluent to afford 2.11 g of product that was used for the next step without further purification.

5-[12-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-dodecyl]-imidazole-1-sulfonic acid dimethylamide (5f)

4f (1.54 g, ± 0.0025 mol) was mixed with isoindole-1,3-dione (1.22 g, 0.0083 mol), Na_2CO_3 (0.87 g, 0.0083 mol) and NaI (0.075 g, 0.50 mmol) in dry DMF (40 mL) and heated at 90 °C. After 20 h the solvent was evaporated under reduced pressure, the residue was diluted with water and extracted with $CHCl_3$. The combined organic extract were washed with water, dried (Na_2SO_4) and concentrated in vacuo to give **5f** as a brown solid (2.07 g) which was used without further purification.

5-(12-Amino-dodecyl)-imidazole-1-sulfonic acid dimethylamide (6f)

5f (2.07 g) was dissolved in warm ethanol (60 mL), hydrazine monohydrate (0.50 g, 0.01 mol) was added and the solution was refluxed for 3 h, cooled to room temperature and filtered. The filtrate was concentrated in vacuo and the residue was diluted with NaOH (1 M), then extracted with $CHCl_3$. The organic extracts were washed with NaOH (0.5 M), dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified by flash chromatography

switching gradually the eluent from ethyl acetate into methanol/triethylamine (9/1) (R_f = 0.5). 340 mg (38%) of a yellow solid was isolated. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 16H, central CH_2's + NH_2), 1.65 (m, 6H, 3 × CH_2), 2.65 (m, 4H, imidazole-5-CH_2 + CH_2NH_2), 2.83 (s, 6H, $N(CH_3)_2$), 6.75 (s, 1H, imidazole-4H), 7.78 (s, 1H, imidazole-2H).

5-(8-Pyrrolidin-1-yl-octyl)-imidazole-1-sulfonic acid dimethylamide (7i)

4i (1.5 g, 0.0047 mol) was stirred at room temperature with pyrrolidine (4.37 g, 0.062 mol). After 48 h the unreacted pyrrolidine was removed under reduced pressure and the residue was diluted with water, basified with K_2CO_3, extracted with dichloromethane, dried (Na_2SO_4) and concentrated in vacuo to afford 1.30 g (78%) of brown oil. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 10H, central CH_2's), 1.75 (m, 6H, 3 × CH_2), 2.35 (m, 6H, 2 × CH_2 + CH_2-pyrrolidine), 2.65 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, $N(CH_3)_2$), 6.75 (s, 1H, imidazole-4H), 7.78 (s, 1H, imidazole-2H).

5-(10-Pyrrolidin-1-yl-decyl)-imidazole-1-sulfonic acid dimethylamide (7j)

The same procedure as for **7i** with 4.43 g (± 0.0029 mol) of **4j** was used. The product was purified by flash chromatography switching gradually the eluent from ethyl acetate into methanol/triethylamine (9/1). 760 mg (67%) of a brown oil was obtained. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 14H, central CH_2's), 1.75 (m, 6H, 3 × CH_2), 2.50 (m, 6H, 2 × CH_2 + CH_2-pyrrolidine), 2.65 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, $N(CH_3)_2$), 6.85 (s, 1H, imidazole-4H), 7.90 (s, 1H, imidazole-2H).

5-(12-Pyrrolidin-1-yl-dodecyl)-imidazole-1-sulfonic acid dimethylamide (7k)

Using the same procedure as for **7j** starting with **4f** (1.00 g, ± 0.0016 mol) gave 500 mg (74%) of a brown oil. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 16H, central CH_2's), 1.75 (m, 8H, 4 × CH_2), 2.45 (m, 6H, 2 × CH_2 + CH_2-pyrrolidine), 2.70 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, $N(CH_3)_2$), 6.80 (s, 1H, imidazole-4H), 7.80 (s, 1H, imidazole-2H).

14-[4-(Toluene-4-sulfonyl)-4,5-dihydro-oxazol-5-yl]-tetradecanoic acid methyl ester (11)

To a stirred suspension of tosyl methyl isocyanide (TosMIC) (4.98 g, 0.026 mol) and 15-oxo-pentadecanoic acid methyl ester **10**[71] (6.99 g, 0.026 mol) in absolute ethanol (80 mL) was added finely powdered sodium cyanide (0.12 g, 0.0026 mol). The yellow-orange suspension was stirred for 30 m, filtered and washed with ethanol/ether. The light yellow solid was immediately used for the next step. ^{1}H NMR ($CDCl_3$): δ 1.30 (m, 18H, central CH_2's), 1.60 (m, 6H, 3 × CH_2), 2.25 (t, 2H, CH_2COOCH_3), 2.46 (s, 3H, *p*-CH_3), 3.65 (s, 3H, CH_3OCO), 4.76 (d, 1H, oxazoline-4H), 5.05 (q, 1H, oxazoline-5H), 6.95 (s, 1H, oxazoline-2H), 7.35 (d, 2H, 2,6-phenyl-H), 7.80 (d, 2H, 3,5-phenyl-H).

14-(1*H*-Imidazol-4-yl)-tetradecanoic acid amide (12)

In a re-sealable pressure tube, a solution of **11** in a saturated solution of ammonia in dry methanol was heated between 120-140 °C. After 48 h the reaction mixture was cooled to r.t. and the solvent was removed in vacuo. The residue was triturated with dichloromethane (150 mL) and filtered. 4.41 g (58%) of a beige solid was collected. ^{1}H NMR (DMSO): δ 1.25 (m, 18H, central C*H*$_2$'s), 1.50 (m, 4H, 3 × C*H*$_2$), 2.05 (t, 2H, C*H*$_2$CONH$_2$), 2.50 (t, 2H, imidazole-4(5)-C*H*$_2$), 6.70 (s, 1H, imidazole-4(5)H), 7.65 (s, 1H, imidazole-2H).

9-(1*H*-Imidazol-4-yl)-nonylamine Dioxalate (1c)

5c (0.96 g, 0.0022 mol) was dissolved in 30% HBr (12 mL) and heated under reflux. After 16 h the mixture was cooled and concentrated in vacuo, the residue was diluted with water, washed with diethyl ether, basified with K_2CO_3 and extracted with $CHCl_3$/EtOH (9/1). The organic layers were combined, dried ($MgSO_4$) and concentrated in vacuo to afford 0.30 g (65%) of brown solid.

The free base was converted into the dioxalate salt, using the following procedure: the free base was dissolved in ethyl acetate with a few drops of methanol, then added dropwise with a solution of 2 equiv of oxalic acid dihydrate in ethyl acetate/methanol. The precipitate that formed was collected by filtration to afford 0.42 g (77%) of a light yellow powder. Mp: 126-128 °C. ^{1}H NMR (D_2O): δ 1.27 (m, 10H, central C*H*$_2$'s), 1.62 (m, 4H, 2 × C*H*$_2$), 2.67 (t, 2H, imidazole-4(5)-C*H*$_2$), 2.94 (t, 2H, C*H*$_2$NH$_2$), 7.14 (s, 1H, imidazole-4(5)H), 8.50 (s, 1H, imidazole-2H). Anal. ($C_{16}H_{27}N_3O_8$) C, H, N.

11-(1*H*-Imidazol-4-yl)-undecylamine (1e)

Using the same procedure as for **1c** starting with 2.11 g (± 0.0040 mol) of **5e** gave 0.78 g (82%) of light yellow powder. Mp: 87.5-88.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.27 (m, 16H, central C*H*$_2$'s + N*H*$_2$), 1.50 (m, 4H, 2 × C*H*$_2$), 2.60 (m, 4H, imidazole-4(5)-C*H*$_2$ + C*H*$_2$NH$_2$), 6.70 (s, 1H, imidazole-4(5)H), 7.50 (s, 1H, imidazole-2H). Anal. ($C_{14}H_{27}N_3$) C, H, N.

12-(1*H*-Imidazol-4-yl)-dodecylamine (1f)

The same procedure as for **1c** with 0.34 g (0.95 mmol) of **6f** was used. After basification the water was removed in vacuo and the remaining solid was washed with 10 mL portions of 2-propanol. The filtrate was concentrated under reduced pressure and washed with fresh 2-propanol. The filtrates were concentrated in vacuo to give 0.16 g (67%) of a beige powder. Mp: 150-152 °C. ^{1}H NMR ($CDCl_3$): δ 1.20 (m, 16H, central C*H*$_2$'s + N*H*$_2$), 1.55 (m, 6H, 3 × C*H*$_2$), 2.60 (m, 4H, imidazole-4(5)-C*H*$_2$ + C*H*$_2$NH$_2$), 6.70 (s, 1H, imidazole-4(5)H), 7.50 (s, 1H, imidazole-2H). Anal. ($C_{15}H_{29}N_3$) C, H, N.

13-(1*H*-Imidazol-4-yl)-tridecylamine Dioxalate (1g)

PIFA [51] (1.72 g, 0.0040 mol) was dissolved in 6 mL of acetonitrile, and 6 mL of distilled water was added. To this solution was added **12** (1.17 g, 0.0040 mol), and the suspension was stirred at room temperature. After 20 h the reaction mixture was diluted with water (75 mL), HCl (37%, 8 mL), stirred for another 30 m and washed with diethyl ether. The combined ether layers were extracted with HCl (10%, 30 mL). The aqueous layers were combined, basified with K_2CO_3, extracted with chloroform/ethanol (10%), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by flash chromatography with methanol and then methanol/triethylamine (9/1) to afford 240 mg (23%) of amine. The free base was converted into a dioxalate. 335 mg (83%) of a white powder was obtained. Mp: 118-120 °C. ^{1}H NMR (D_2O): δ 1.27 (m, 18H, central C*H*$_2$'s), 1.62 (m, 4H, 2 × C*H*$_2$), 2.67 (t, 2H, imidazole-4(5)-C*H*$_2$), 2.94 (t, 2H, C*H*$_2$NH$_2$), 7.14 (s, 1H, imidazole-4(5)H), 8.50 (s, 1H, imidazole-2H). Anal. ($C_{20}H_{35}N_3O_8$) C, H, N.

14-(1*H*-Imidazol-4-yl)-tetradecylamine Dioxalate (1h)

A suspension of **12** (2.00 g, 0.068 mol) in THF (100 mL) was added, with efficient stirring, to a suspension of $LiAlH_4$ (1.30 g, 0.034 mol) in THF (30 mL). The reaction mixture was stirred for 1 h at r.t., and then heated under reflux. After 20 h the suspension was cooled to r.t. and water (1.5 mL), followed by NaOH 10% aqueous solution (1.5 mL) was added drop wise and refluxed. After 30 m the reaction mixture was cooled to room temperature, filtered and concentrated in vacuo to afford 1.66 g (88%) of a yellow solid that was converted into the dioxalate salt. The yellow precipitate collected by filtration was washed (under stirring) with methanol (10 mL) and filtered again to afford 1.12 g (42%) of a light-yellow powder. Mp: 122-126 °C. ^{1}H NMR (D_2O): δ 1.27 (m, 20H, central C*H*$_2$'s), 1.62 (m, 4H, 2 × C*H*$_2$), 2.67 (t, 2H, imidazole-4(5)-C*H*$_2$), 2.94 (t, 2H, C*H*$_2$NH$_2$), 7.14 (s, 1H, imidazole-4(5)H), 8.50 (s, 1H, imidazole-2H). Anal. ($C_{21}H_{37}N_3O_8$) C, H, N.

4-(8-Pyrrolidin-1-yl-octyl)-1*H*-imidazole Dioxalate (1i)

Using the same procedure as for **1c** with **7i** (1.25 g, 0.0035 mol) gave 800 mg (92%) of product. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 8H, central C*H*$_2$'s), 1.50 (m, 4H, 2 × C*H*$_2$), 1.75 (m, 4H, 2 × C*H*$_2$), 2.45 (m, 8H, imidazole-4(5)-C*H*$_2$ + C*H*$_2$-pyrrolidine + 2 × C*H*$_2$), 6.70 (s, 1H, imidazole-4(5)H), 7.45 (s, 1H, imidazole-2H). The free base was converted into the dioxalate salt and crystallized from methanol/diethyl ether to afford 150 mg (11%) of light yellow solid. Anal. ($C_{19}H_{31}N_3O_8$) C, H, N.

4-(10-Pyrrolidin-1-yl-decyl)-1*H*-imidazole (1j)

Using the same procedure as for **1c** with **7j** (0.76 g, 0.0020 mol), followed by the treatment of the free base with activated charcoal, gave 480 mg (87%) of a brown waxy compound. ^{1}H

NMR ($CDCl_3$): δ 1.20 (m, 12H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 1.70 (m, 4H, 2 × CH_2), 2.45 (m, 8H, imidazole-4(5)-CH_2 + CH_2-pyrrolidine + 2 × CH_2), 6.70 (s, 1H, imidazole-4(5)H), 7.45 (s, 1H, imidazole-2H). Anal. ($C_{17}H_{31}N_3$) C, H, N.

4-(12-Pyrrolidin-1-yl-dodecyl)-1*H*-imidazole (1k)

Using the same procedure as for **1j** gave 210 mg (57%) of a waxy yellow solid. ^1H NMR ($CDCl_3$): δ 1.20 (m, 16H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 1.70 (m, 4H, 2 × CH_2), 2.45 (m, 8H, imidazole-4(5)-CH_2 + CH_2-pyrrolidine + 2 × CH_2), 6.70 (s, 1H, imidazole-4(5)H), 7.45 (s, 1H, imidazole-2H). Anal. ($C_{19}H_{35}N_3$) C, H, N.

2-(9-Bromo-nonyl)-pyridine (15a)

0.54 g (0.0058 mol) of 2-methyl-pyridine **14a** in THF (5 mL) was added drop wise to a solution of *n*-butyllithium (3.65 mL, 0.0058 mol) in 5 mL of THF at −50 °C over 10 m, then the cooling bath was removed and the temperature was allowed to rise to −20 °C. The cold dark red solution was added drop wise to a cold solution (-50 °C) of dibromooctane (2.44 g, 0.0090 mol) in THF (5 mL) and the mixture was allowed to warm to room temperature and stirred for an additional ½ h. HCl (37%) was added till pH = 1 and stirred for 1 ½ h, then the solvent was removed in vacuo. The residue was washed with diethyl ether, basified with K_2CO_3, extracted with dichloromethane, dried ($MgSO_4$) and evaporated in vacuo. The residue (0.82 g) was used directly for the next step.

10-Pyridin-2-yl-decan-1-ol (15b)

5.63 g (0.061 mol) of **14a** in THF (30 mL) was added drop wise to a solution of *n*-butyllithium (37.8 mL, 0.061 mol) in 50 mL of THF at −50 °C over 10 m, then the cooling bath was removed and the temperature allowed to rise to −20 °C. The red solution was cooled again to −50 °C and 16.77 g (0.055 mol) of 2-(9-bromo-nonyloxy)-tetrahydro-pyran in THF (30 mL) was added dropwise, then the mixture was allowed to (slowly) warm to room temperature overnight. The reaction mixture was poured into water and the THF was removed under reduced pressure. The residue was acidified with HCl (37%) and stirred for 30 m, washed with diethyl ether, basified with K_2CO_3, extracted with dichloromethane, dried ($MgSO_4$) and evaporated in vacuo. The product was purified by flash chromatography switching gradually the eluent from dichloromethane/ethyl acetate (9/1) into ethyl acetate. 4.49 g (35%) of a white waxy compound was isolated. ^1H NMR ($CDCl_3$): δ 1.15 (m, 12H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 2.60 (t, 2H, pyridine-2-CH_2), 2.98 (br s*, 1H, O*H*), 3.45 (t, 2H, CH_2OH), 6.90 (m, 2H, pyridine-3H + pyridine-5H), 7.38 (m, 1H, pyridine-4H), 8.30 (d, 1H, pyridine-6H).

12-Pyridin-2-yl-dodecan-1-ol (15c)

The same procedure as for **15b** with 12.35 g (0.037 mol) of 2-(11-bromo-undecyloxy)-tetrahydro-pyran was used. After the basification the product precipitated and was filtered and washed with water to afford 5.67 g (58%) of a beige powder. ^{1}H NMR ($CDCl_3$): δ 1.23 (m, 14H, central CH_2's), 1.60 (m, 6H, 3 × CH_2), 2.75 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.05 (m, 2H, pyridine-3H + pyridine-5H), 7.55 (m, 1H, pyridine-4H), 8.45 (d, 1H, pyridine-6H).

10-Pyridin-3-yl-decan-1-ol (15d)

A solution of 2.89 g (0.031 mol) of 3-methyl pyridine **14d** in THF (20 mL) was added dropwise at −20 °C to a freshly prepared solution of lithium diisopropylamide (19.38 mL, 0.031 mol of n-butyllithium and 3.74 g, 0.037 mol of diisopropylamine) in 70 mL of THF. The cooling bath was removed and the temperature allowed to rise to +10 °C. After stirring for 30 m at this temperature the reaction mixture was cooled to −40 °C and 8.00 g (0.026 mol) of 2-(9-bromo-nonyloxy)-tetrahydro-pyran in THF (30 mL) was added dropwise. The cooling bath was removed and the mixture was allowed to (slowly) warm to room temperature overnight. The reaction mixture was poured into water and the THF was removed under reduced pressure. The crude product was acidified with HCl (37%) and stirred for 30 m, washed with diethyl ether (3 × 50 mL), basified with K_2CO_3, extracted with dichloromethane, dried ($MgSO_4$) and evaporated in vacuo, to afford 5.50 g (90%) of a brown waxy compound. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 12H, central CH_2's), 1.52 (m, 4H, 2 × CH_2), 2.58 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.15 (m, 1H, pyridine-5H), 7.45 (d, 1H, pyridine-4H), 8.38 (m, 2H, pyridine-2H + pyridine-6H).

12-Pyridin-3-yl-dodecan-1-ol (15e)

Using the same procedure as for **15d** with 1.78 g (0.019 mol) of **14d** and 5.36 g (0.016 mol) of 2-(11-bromo-undecyloxy)-tetrahydro-pyran gave 2.56 g (61%) of a brown waxy compound. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 16H, central CH_2's), 1.55 (m, 6H, 3 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.15 (m, 1H, pyridine-5H), 7.45 (d, 1H, pyridine-4H), 8.38 (m, 2H, pyridine-2H + pyridine-6H).

10-Pyridin-4-yl-decan-1-ol (15f)

The same procedure as for the preparation of **15d** was employed, using 3.02 g (0.032 mol) of 4-methyl pyridine **14f**, with the exception that **14f** solution was added at −40 °C and the reaction mixture was stirred for 30 m at +20 °C. Upon addition of HCl (37%) a white precipitate formed, that was filtered. The solid was dissolved in water, basified with K_2CO_3 and filtered to afford 4.89 g (77%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 12H, central CH_2's), 1.55 (m, 4H, 2 × CH_2), 1.85 (br s, 1H, O*H*), 2.55 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.10 (d, 2H, pyridine-3H + pyridine-5H), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

12-Pyridin-4-yl-dodecan-1-ol (15g)

Using the same procedure as for **15f** with 1.78 g (0.019 mol) of **14f** gave 3.67 g (87%) of a brown oil. ^{1}H NMR ($CDCl_3$): δ 1.25 (m, 14H, central CH_2's), 1.55 (m, 6H, 3 × CH_2), 1.75 (br s, 1H, O*H*), 2.55 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.10 (d, 2H, pyridine-3H + pyridine-5H), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

2-(10-Pyridin-2-yl-decyl)-isoindole-1,3-dione (16b)

The same procedure as for **5c** with 4.49 g (0.019 mol) of **15b** was used. After removal of the solvent the residue was dissolved in ethyl acetate and extracted with HCl (1 M). The aqueous layer was washed once with ethyl acetate, basified with K_2CO_3, extracted with $CHCl_3$, dried ($MgSO_4$) and evaporated to afford 6.25 g (90%) of white powder. ^{1}H NMR ($CDCl_3$): δ 1.23 (m, 12H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.75 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-N), 7.05 (m, 2H, pyridine-3H + pyridine-5H), 7.50 (m, 1H, pyridine-4H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (d, 1H, pyridine-6H).

2-(12-Pyridin-2-yl-dodecyl)-isoindole-1,3-dione (16c)

The same procedure as for **16b** with 5.67 g (0.022 mol) of **15c** was used. During the extraction with HCl (1 M) the hydrochloric salt of the compound crystallized and was separated by filtration. The salt was dissolved in water, basified with K_2CO_3, extracted with $CHCl_3$, dried ($MgSO_4$) and concentrated in vacuo to afford 3.19 g (38%) of white powder. ^{1}H NMR ($CDCl_3$): δ 1.23 (m, 14H, central CH_2's), 1.60 (m, 6H, 3 × CH_2), 2.75 (t, 2H, pyridine-2-CH_2), 3.65 (t, 2H, CH_2-N), 7.05 (m, 2H, pyridine-3H + pyridine-5H), 7.55 (m, 1H, pyridine-4H), 7.68 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (d, 1H, pyridine-6H).

2-(10-Pyridin-3-yl-decyl)-isoindole-1,3-dione (16d)

Using the same procedure as for **16b** with 5.50 g (0.023 mol) of **15d** gave 7.57 g (90%) of brown oil. ^{1}H NMR ($CDCl_3$): δ 1.23 (m, 12H, central CH_2's), 1.55 (m, 4H, 2 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 7.15 (m, 2H, pyridine-3H + pyridine-5H), 7.15 (m, 1H, pyridine-5H), 7.45 (d, 1H, pyridine-4H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.38 (m, 2H, pyridine-2H + pyridine-6H).

2-(10-Pyridin-4-yl-decyl)-isoindole-1,3-dione (16f)

Using the same procedure as for **16b** with 4.89 g (0.021 mol) of **15f** gave 6.42 g (82%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.23 (m, 12H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 7.05 (d, 2H, pyridine-3H + pyridine-5H), 7.45 (d, 1H, pyridine-4H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

2-(12-Pyridin-4-yl-dodecyl)-isoindole-1,3-dione (16g)

Using the same procedure as for **16b** with 3.67 g (0.014 mol) of **15g** gave 4.37 g (79%) of brown oil. ^{1}H NMR ($CDCl_3$): δ 1.23 (m, 14H, central CH_2's), 1.55 (m, 6H, 3 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 7.05 (d, 2H, pyridine-3H + pyridine-5H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

9-Pyridin-2-yl-nonylamine Dihydrochloride (13a)

The same procedure as for **5f** with 0.82 g of crude **15a** was used. The residue obtained after extraction was purified by flash chromatography using ethyl acetate as eluent. The product fractions were hydrazinolysed as described for **6f** to give 0.12 g (59%) of free base.

The free base was converted into the dihydrochloride, using the following procedure: the free base was mixed with 2 equiv of HCl (37%) and stirred for 30 m, then the water was removed in vacuo to afford 0.15 g (95%) of a brown waxy compound. ^{1}H NMR (D_2O): δ 1.25 (m, 10H, central CH_2's), 1.70 (m, 6H, 2 × CH_2 + NH_2), 3.00 (m, 4H, pyridine-2-CH_2 + CH_2NH_2), 7.85 (m, 2H, pyridine-3H + pyridine-5H), 8.43 (m, 1H, pyridine-4H), 8.55 (d, 1H, pyridine-6H). Anal. ($C_{14}H_{26}Cl_2N_2$) C, H, N.

10-Pyridin-2-yl-decylamine Dihydrochloride (13b)

Using the same procedure as for **6f** with 6.25 g (0.017 mol) of **16b** gave 2.99 g (74%) of free base. The free base was converted into the dihydrochloride, decolorized with activated charcoal and crystallized from ethanol/ethyl acetate to afford 2.18 g (56%) of white crystals. Mp: 143.5-146.5 °C. ^{1}H NMR (D_2O): δ 1.25 (m, 14H, central CH_2's + NH_2), 1.70 (m, 4H, 2 × CH_2), 3.00 (m, 4H, pyridine-2-CH_2 + CH_2NH_2), 7.85 (m, 2H, pyridine-3H + pyridine-5H), 8.43 (m, 1H, pyridine-4H), 8.55 (d, 1H, pyridine-6H). Anal.($C_{15}H_{28}Cl_2N_2$) C, H, N.

12-Pyridin-2-yl-dodecylamine Dihydrochloride (13c)

Using the same procedure as for **13b** with 3.19 g (0.0081 mol) of **16c** gave 1.65 g (66%) of white crystals. Mp: 161-164 °C. ^{1}H NMR (D_2O): δ 1.25 (m, 16H, central CH_2's), 1.70 (m, 4H, 2 × CH_2), 3.00 (m, 4H, pyridine-2-CH_2 + CH_2NH_2), 7.85 (m, 2H, pyridine-3H + pyridine-5H), 8.48 (m, 1H, pyridine-4H), 8.58 (d, 1H, pyridine-6H). Anal. ($C_{17}H_{32}Cl_2N_2$) C, H, N.

10-Pyridin-3-yl-decylamine Dihydrochloride (13d)

The same procedure as for **6f** was employed starting with 7.57 g (0.021 mol) of **16d**. The free base obtained after extraction was converted into the dihydrochloride and crystallized from ethanol/ethyl acetate to afford 1.78 g (28%) of a beige powder. Mp: 93.5-96.5 °C. ^{1}H NMR (D_2O): δ 1.30 (m, 12H, central CH_2's), 1.68 (m, 4H, 2 × CH_2), 2.82 (t, 2H, pyridine-2-CH_2),

2.95 (t, 2H, CH_2NH_2), 7.95 (m, 1H, pyridine-5H), 8.45 (d, 1H, pyridine-4H), 8.60 (m, 2H, pyridine-2H + pyridine-6H). Anal. ($C_{15}H_{28}Cl_2N_2$) C, H, N.

12-Pyridin-3-yl-dodecylamine Dihydrochloride (13e)

The same procedure as for **5c** with 2.56 g (0.0098 mol) of **15e** was used. The solvents were removed in vacuo and the remaining oil was hydrazinolysed using the same procedure as for **6f**. The free base was converted into the dihydrochloride and crystallized from ethanol/ethyl acetate to afford 1.32 g (40%) of a yellow powder. Mp: 110-112 °C. ^{1}H NMR (D_2O): δ 1.30 (m, 18H, central CH_2's + NH_2), 1.68 (m, 4H, 2 × CH_2), 2.82 (t, 2H, pyridine-2-CH_2), 2.95 (t, 2H, CH_2NH_2), 7.95 (m, 1H, pyridine-5H), 8.45 (d, 1H, pyridine-4H), 8.60 (m, 2H, pyridine-2H + pyridine-6H). Anal. ($C_{17}H_{32}Cl_2N_2$) C, H, N.

10-Pyridin-4-yl-decylamine Dihydrochloride (13f)

The same procedure as for **6f** with 6.42 g (0.018 mol) of **16f** was used. The free base was converted into the dihydrochloride, crystallized from ethanol/ethyl acetate and decolorized with activated charcoal to afford 2.65 g (49%) of light yellow powder. Mp: 143.5-144.5 °C. ^{1}H NMR (D_2O): δ 1.25 (m, 14H, central CH_2's + NH_2), 1.65 (m, 4H, 2 × CH_2), 2.95 (m, 4H, pyridine-2-CH_2 + CH_2NH_2), 7.85 (d, 2H, pyridine-3H + pyridine-5H), 8.58 (d, 2H, pyridine-2H + pyridine-6H). Anal. ($C_{15}H_{28}Cl_2N_2$) C, H, N.

12-Pyridin-4-yl-dodecylamine Dihydrochloride (13g)

The same procedure as for **6f** with 4.37 g (0.011 mol) of **16g** was used. The crude product was purified by flash chromatography switching gradually the eluent from ethyl acetate into methanol/triethylamine (9/1) (R_f = 0.4) to afford 1.48 g (50%) of yellow solid. The free base was converted into the dihydrochloride, crystallized from ethanol/ethyl acetate and decolorized with activated charcoal to afford 1.28 g (68%) of light yellow powder. Mp: 137.5-139.5 °C. ^{1}H NMR (D_2O): δ 1.25 (m, 18H, central CH_2's + NH_2), 1.65 (m, 4H, 2 × CH_2), 2.95 (m, 4H, pyridine-2-CH_2 + CH_2NH_2), 7.85 (d, 2H, pyridine-3H + pyridine-5H), 8.58 (d, 2H, pyridine-2H + pyridine-6H). Anal. ($C_{17}H_{32}Cl_2N_2$) C, H, N.

Table 5. Elemental analyses.

			C		H		N	
Compound	Code	Formula	Calc.	Found	Calc.	Found	Calc.	Found
1c	VUF 5695	$C_{16}H_{27}N_3O_8$	49.35	49.62	10.79	10.92	6.99	7.04
1e	VUF 5696	$C_{14}H_{27}N_3$	70.83	70.88	17.70	17.59	11.46	11.43
1f	VUF 5671	$C_{15}H_{29}N_3$	71.66	71.53	16.71	16.67	11.63	11.61
1g	VUF 5697	$C_{20}H_{35}N_3O_8$	53.92	53.59	9.43	9.34	7.92	7.88
1h	VUF 5673	$C_{21}H_{37}N_3O_8$	54.89	54.79	9.14	8.75	8.12	8.18
1i	VUF 5669	$C_{19}H_{31}N_3O_8$	53.14	52.86	9.78	9.68	7.28	7.22
1j	VUF 5670	$C_{17}H_{31}N_3$	73.59	73.15	15.15	15.03	11.26	11.20
1k	VUF 5672	$C_{19}H_{35}N_3$	74.70	74.31	13.75	13.61	11.55	11.58
13a	VUF 5680	$C_{14}H_{26}Cl_2N_2$	57.34	56.99	9.55	9.49	8.94	8.95
13b	VUF 5674	$C_{15}H_{28}Cl_2N_2$	58.63	58.56	9.12	9.10	9.18	9.18
13c	VUF 5677	$C_{17}H_{32}Cl_2N_2$	60.89	60.83	8.35	8.30	9.62	9.64
13d	VUF 5675	$C_{15}H_{28}Cl_2N_2$	58.63	58.53	9.12	9.09	9.18	9.49
13e	VUF 5678	$C_{17}H_{32}Cl_2N_2$	60.89	60.79	8.35	8.34	9.62	9.62
13f	VUF 5676	$C_{15}H_{28}Cl_2N_2$	58.63	58.53	9.12	8.89	9.18	9.24
13g	VUF 5679	$C_{17}H_{32}Cl_2N_2$	60.89	60.98	8.35	8.56	9.62	9.69

Pharmacology

Materials. pNF-κB-Luc was obtained from Stratagene (La Jolla, USA). Mepyramine (pyrilamine maleate), tripelennamine hydrochloride were obtained from RBI (USA). ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, polyethyleneimine, and Tween 20 were purchased from Sigma Chemical Company (USA). D-luciferin was obtained from Duchefa Biochemie BV (Haarlem, The Netherlands), glycerol from Riedel-de-Haën (Germany), and Triton X-100 from Fluka (Switzerland). Cell culture media, penicillin, and streptomycin were obtained from Life Technologies (Merelbeke, Belgium). Foetal calf serum (FCS) was obtained from Integro B.V. (Dieren, The Netherlands), dialysed foetal calf serum was obtained from HyClone® Laboratories Inc. (USA). [^{3}H]Mepyramine (30 Ci/mmol), *myo*-[2-^{3}H]inositol (17 Ci/mmol), were from Amersham International (United Kingdom).

All other compounds, including VUF 4732 (**1a**), VUF 4733 (**1b**), VUF 4734 (**1d**), [53] VUF 8811 (**17c**), VUF 5963 (**17a**), 2-(pyridine-2-yl)ethyl-1-amine (PEA) dihydrochloride, 2-(thiazol-4-yl)ethanamine (4-TEA), 2-(3-trifluoromethylphenyl)histamine dihydrogenmaleate (**17b**),[56] histaprodifen dimaleate (**18c**), histabudifen dimaleate (**18d**), histapendifen dimaleate (**18e**), the pyrrolidin-analogue of histaprodifen as dioxalate (**18i**),[58] the ring-substituted analogues of histaprodifen dihydrogenmaleate [4-Br-histaprodifen (**18g**) and 4-methyl-histaprodifen (**18h**)],[59] the histaprodifen analogues 2-(diphenylmethyl)histamine dihydrochloride (**18a**) and 2-(diphenylethyl)histamine dihydrochloride (**18b**), 2-(4-phenylbutyl)histamine dihydrogenmaleate (FUB 113, **17d**), 2-(5-phenylpentyl)histamine dihydrogenmaleate (FUB 114, **17e**), the cyclohexyl-analogue of histaprodifen dihydrogenmaleate (**18f**),[55] the 2-(2-

phenyl-4-thiazolyl)ethyl-1-amines (**19a-b**) and 2-(2-benzyl-4-thiazolyl)ethyl-1-amines (**19c-d**)[60] are taken from our own stock.

Gifts of mianserine hydrochloride (Organon NV, the Netherlands), $pcDEF_3$ (Dr. J. Langer), and of the cDNA encoding the human histamine H_1 receptor (Dr. H.Fukui)[2] are greatly acknowledged.

Cell culture and transfection. COS-7 African green monkey kidney cells were maintained at 37 °C in an humidified 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin and 5% (v/v) fetal calf serum (FCS); HeLa cells were maintained in the same manner in medium containing 10% (v/v) FCS. COS-7 cells were transiently transfected using the DEAE-dextran method as previously described.[28] The expression level of the histamine H_1 receptor in transiently transfected COS-7 cells was 3.2 ± 0.4 pmol/mg protein as determined by radioligand binding assays. The total amount of DNA transfected was maintained constant by addition of either $pcDEF_3$ or $pcDNA_3$.

Reporter-gene assay. Reporter-gene assays were preformed as previously described.[28] Briefly, after co-transfection with pNFκB-Luc (125 μg/$1 \cdot 10^7$ cells) and either $pcDEF_3$ or $pcDEF_3hH_1$ (25 μg/$1 \cdot 10^7$ cells) cells were seeded in 96 well whiteplates in serum free culture medium and incubated with drugs. After 48 h, cells were assayed for luminescence for 3 sec/well in a Victor^2 (Wallac) 30 m after aspiration of the medium and the addition of 25 μL/well luciferase assay reagent (0.83 mM ATP, 0.83 mM d-luciferin, 18.7 mM $MgCl_2$, 0.78 μM $Na_2H_2P_2O_7$, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton-X-100 and 2.6 μM DTT). To detect potential non-human H_1R-mediated effects of the tested compounds, the compounds were tested in parallel on control cells not expressing the human H_1R.

Histamine H_1 receptor binding studies. HeLa cells or COS-7 cells (harvested 48 h after transfection) for radioligand binding studies were harvested and homogenised in ice-cold H_1-binding buffer (50 mM Na_2/K-phosphate buffer, pH = 7.4) as previously described.[28] HeLa cell membranes were further purified by centrifugation of the cell homogenate for 5 m at 1,400 x g, and subsequent centrifugation of the supernatant for 20 m at 15,000 x g. For radioligand binding studies, COS-7 cell homogenates or HeLa cell membranes were incubated for 30 min at 30°C in H_1-binding buffer containing 1 nM [^{3}H]mepyramine. The non-specific binding was determined in the presence of 1 μM mianserin. Incubations were terminated by rapid dilution and subsequent filtration over Whatman GF/C filters that had been treated with 0.3% polyethyleneimine using ice-cold H_1-binding buffer. The radioactivity retained on the filters was measured by liquid scintillation counting. Binding data were evaluated by a non-linear, least

squares curve-fitting procedure using Graphpad Prism® (GraphPad Software, Inc., San Diego, CA).

[^{3}H]Inositol phosphate formation. HeLa cells were seeded in 24-well plates (10^5 cells/well) and after 24 h cells were washed with PBS, and labelled and pre-treated with various concentrations of inverse H_1 agonists in inositol-free culture medium supplemented with 1 μCi/mL *myo*-[2-^{3}H]inositol for 24h. Subsequently, the medium was aspirated and cells were incubated with histamine for 1 hr at 37 °C in DMEM containing 25mM Hepes (pH 7.4) and 20 mM LiCl. Incubations were stopped by aspiration of the culture medium and the addition of cold 10 mM formic acid. After 90 min incubation at 4 °C, [^{3}H]inositol phosphates were isolated by anion exchange chromatography, and counted by liquid scintillation.

Analytical methods. Protein levels were determined according to Bradford,[72] using BSA as a standard. All data shown are expressed as mean ± S.E.M., statistical analyses were carried out by Student's *t*-test. *P* values < 0.05 were considered to indicate a significant difference.

References

(1) De Backer, M. D.; Gommeren, W.; Moereels, H.; Nobels, G.; van Gompel, P.; Leysen, J. E.; Luyten, W. H. Genomic cloning, heterologous expression and pharmacological characterization of a human histamine H_1 receptor. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 1601-1608.

(2) Fukui, H.; Fujimoto, K.; Mizuguchi, H.; Sakamoto, K.; Horio, Y.; Takai, S.; Yamada, K.; Ito, S. Molecular cloning of the human histamine H_1 receptor gene. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 894-901.

(3) Moguilevsky, N.; Varsalona, F.; Noyer, M.; Gillard, M.; Guillaume, J. P.; Garcia, L.; Szpirer, C.; Szpirer, J.; Bollen, A. Stable expression of human H_1-histamine-receptor cDNA in Chinese hamster ovary cells. Pharmacological characterisation of the protein, tissue distribution of messenger RNA and chromosomal localisation of the gene. *Eur. J. Biochem.* **1994**, *224*, 489-495.

(4) Gantz, I.; Munzert, G.; Tashiro, T.; Schaffer, M.; Wang, L.; Del Valle, J.; Yamada, T. Molecular cloning of the human histamine H_2 receptor. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 1386-1392.

(5) Lovenberg, T. W.; Roland, B. L.; Wilson, S. J.; Jiang, X.; Pyati, J.; Huvar, A.; Jackson, M. R.; Erlander, M. G. Cloning and functional expression of the human histamine H_3 receptor. *Mol. Pharmacol.* **1999**, *55*, 1101-1107.

(6) Zhu, Y.; Michalovich, D.; Wu, H. L.; Tan, K. B.; Dytko, G. M.; Mannan, I. J.; Boyce, R.; Alston, J.; Tierney, L. A.; Li, X.; Herrity, N. C.; Vawter, L.; Sarau, H. M.; Ames, R. S.; Davenport, C. M.; Hieble, J. P.; Wilson, S.; Bergsma, D. J.; Fitzgerald, L. R. Cloning, Expression, and Pharmacological Characterization of a Novel Human Histamine Receptor. *Mol. Pharmacol.* **2001**, *59*, 434-441.

(7) Liu, C.; Ma, X. J.; Jiang, X.; Wilson, S. J.; Hofstra, C. L.; Blevitt, J.; Pyati, J.; Li, X.; Chai, W.; Carruthers, N.; Lovenberg, T. W. Cloning and Pharmacological Characterization of a Fourth Histamine Receptor (H_4) Expressed in Bone Marrow. *Mol. Pharmacol.* **2001**, *59*, 420-426.

(8) Oda, T.; Morikawa, N.; Saito, Y.; Masuho, Y.; Matsumoto, S. Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J. Biol. Chem.* **2000**, *275*, 36781-36786.
(9) Nakamura, T.; Itadani, H.; Hidaka, Y.; Ohta, M.; Tanaka, K. Molecular cloning and characterization of a new human histamine receptor, HH4R. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 615-620.
(10) Morse, K. L.; Behan, J.; Laz, T. M.; West, R. E.; Greenfeder, S. A.; Anthes, J. C.; Umland, S.; Wan, Y.; Hipkin, R. W.; Gonsiorek, W.; Shin, N.; Gustafson, E. L.; Qiao, X.; Wang, S.; Hedrick, J. A.; Greene, J.; Bayne, M.; Monsma, F. J. Cloning and Characterization of a Novel Human Histamine Receptor. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 1058-1066.
(11) Dale, H. H.; Laidlaw, P. P. The Physiological Action of b-imidazolylethylamine. *J. Physiol.* **1910**, *41*, 418-444.
(12) Zhang, M.-Q.; Leurs, R.; Timmerman, H. Histamine H_1-receptor antagonists. *Burger's Medicinal Chemistry and Drug Discovery,*; 5 ed.; John Wiley & Sons, Inc., 1997; pp 495-559.
(13) Costa, T.; Ogino, Y.; Munson, P. J.; Onaran, H. O.; Rodbard, D. Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol. Pharmacol.* **1992**, *41*, 549-560.
(14) Schutz, W.; Freissmuth, M. Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol. Sci.* **1992**, *13*, 376-380.
(15) Lefkowitz, R. J.; Cotecchia, S.; Samama, P.; Costa, T. Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.* **1993**, *14*, 303-307.
(16) Milligan, G.; Bond, R. A.; Lee, M. Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol. Sci.* **1995**, *16*, 10-13.
(17) Kenakin, T. The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol. Rev.* **1996**, *48*, 413-463.
(18) Leurs, R.; Smit, M. J.; Alewijnse, A. E.; Timmerman, H. Agonist-independent regulation of constitutively active G-protein-coupled receptors. *Trends Biochem. Sci.* **1998**, *23*, 418-422.
(19) Smit, M. J.; Leurs, R.; Alewijnse, A. E.; Blauw, J.; van Nieuw Amerongen, G. P.; van De Vrede, Y.; Roovers, E.; Timmerman, H. Inverse agonism of histamine H_2 antagonist accounts for upregulation of spontaneously active histamine H_2 receptors. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 6802-6807.
(20) Wieland, K.; Bongers, G.; Yamamoto, Y.; Hashimoto, T.; Yamatodani, A.; Menge, W. M. B. P.; Timmerman, H.; Lovenberg, T. W.; Leurs, R. Constitutive Activity of Histamine H_3 Receptors Stably Expressed in SK-N-MC Cells: Display of Agonism and Inverse Agonism by H_3 Antagonists. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 908-914.
(21) Morisset, S.; Rouleau, A.; Ligneau, X.; Gbahou, F.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Schwartz, J. C.; Arrang, J. M. High constitutive activity of native H_3 receptors regulates histamine neurons in brain. *Nature (London)* **2000**, *408*, 860-864.
(22) Shenker, A.; Laue, L.; Kosugi, S.; Merendino, J. J., Jr.; Minegishi, T.; Cutler, G. B., Jr. A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature (London)* **1993**, *365*, 652-654.
(23) Robinson, P. R.; Cohen, G. B.; Zhukovsky, E. A.; Oprian, D. D. Constitutively active mutants of rhodopsin. *Neuron* **1992**, *9*, 719-725.
(24) Parma, J.; Duprez, L.; Van Sande, J.; Cochaux, P.; Gervy, C.; Mockel, J.; Dumont, J.; Vassart, G. Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature (London)* **1993**, *365*, 649-651.
(25) Robbins, L. S.; Nadeau, J. H.; Johnson, K. R.; Kelly, M. A.; Roselli-Rehfuss, L.; Baack, E.; Mountjoy, K. G.; Cone, R. D. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell* **1993**, *72*, 827-834.

(26) Spiegel, A. M. Defects in G protein-coupled signal transduction in human disease. *Annu. Rev. Physiol.* **1996**, *58*, 143-170.
(27) Bakker, R. A.; Wieland, K.; Timmerman, H.; Leurs, R. Constitutive activity of the histamine H_1 receptor reveals inverse agonism of histamine H_1 receptor antagonists. *Eur. J. Pharmacol.* **2000**, *387*, R5-R7.
(28) Bakker, R. A.; Schoonus, S.; Smit, M. J.; Timmerman, H.; Leurs, R. Histamine H_1-receptor activation of NF-kB: roles for Gbg and $Ga_{q/11}$-subunits in constitutive and agonist-mediated signaling. *Mol. Pharmacol.* **2001**, *60*, 1133-1142.
(29) Costa, T.; Herz, A. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 7321-7325.
(30) Senogles, S. E.; Spiegel, A. M.; Padrell, E.; Iyengar, R.; Caron, M. G. Specificity of receptor-G protein interactions. Discrimination of Gi subtypes by the D_2 dopamine receptor in a reconstituted system. *J. Biol. Chem.* **1990**, *265*, 4507-4514.
(31) Munshi, R.; Pang, I. H.; Sternweis, P. C.; Linden, J. A_1 adenosine receptors of bovine brain couple to guanine nucleotide- binding proteins G_{i1}, G_{i2}, and G_o. *J. Biol. Chem.* **1991**, *266*, 22285-22289.
(32) Burstein, E. S.; Spalding, T. A.; Brann, M. R. Pharmacology of muscarinic receptor subtypes constitutively activated by G proteins. *Mol. Pharmacol.* **1997**, *51*, 312:319.
(33) Burstein, E. S.; Spalding, T. A.; Bräuner-Osborne, H.; Brann, M. R. Constitutive activation of muscarinic receptors by the G-protein G_q. *FEBS Lett.* **1995**, *363*, 261-263.
(34) Casarosa, P.; Bakker, R. A.; Verzijl, D.; Navis, M.; Timmerman, H.; Leurs, R.; Smit, M. J. Constitutive Signaling of the Human Cytomegalovirus-encoded Chemokine Receptor US28. *J. Biol. Chem.* **2001**, *276*, 1133-1137.
(35) Samama, P.; Cotecchia, S.; Costa, T.; Lefkowitz, R. J. A mutation-induced activated state of the b_2-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.* **1993**, *268*, 4625-4636.
(36) Allen, L. F.; Lefkowitz, R. J.; Caron, M. G.; Cotecchia, S. G-protein-coupled receptor genes as protooncogenes: constitutively activating mutation of the a_{1B}-adrenergic receptor enhances mitogenesis and tumorigenicity. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 11354-11358.
(37) Dosil, M.; Giot, L.; Davis, C.; Konopka, J. B. Dominant-negative mutations in the G-protein-coupled alpha-factor receptor map to the extracellular ends of the transmembrane segments. *Mol. Cell. Biol.* **1998**, *18*, 5981-5991.
(38) Chen, S.; Lin, F.; Xu, M.; Hwa, J.; Graham, R. M. Dominant-negative activity of an a_{1B}-adrenergic receptor signal-inactivating point mutation. *EMBO J.* **2000**, *19*, 4265-4271.
(39) Iriyoshi, N.; Takeuchi, K.; Yuta, A.; Ukai, K.; Sakakura, Y. Increased expression of histamine H_1 receptor mRNA in allergic rhinitis. *Clin. Exp. Allergy* **1996**, *26*, 379-385.
(40) Chiba, Y.; Saitoh, N.; Matsuo, K.; Misawa, M. Elevated nasal mucosal G protein levels and histamine receptor affinity in a guinea pig model of nasal hyperresponsiveness. *Int. Arch. Allergy Immunol.* **2002**, *127*, 285-293.
(41) Milligan, G.; Bond, R. A. Inverse agonism and the regulation of receptor number. *Trends Pharmacol. Sci.* **1997**, *18*, 468-474.
(42) Elfellah, M. S.; Reid, J. L. Regulation of b_1- and b_2-adrenoceptors following chronic treatment with b-adrenoceptor antagonists. *Eur. J. Pharmacol.* **1989**, *173*, 85-92.
(43) Homcy, C. J.; Vatner, S. F.; Vatner, D. E. Beta-adrenergic receptor regulation in the heart in pathophysiologic states: abnormal adrenergic responsiveness in cardiac disease. *Annu. Rev. Physiol.* **1991**, *53*, 137-159.
(44) Yoburn, B. C.; Duttaroy, A.; Shah, S.; Davis, T. Opioid antagonist-induced receptor upregulation: effects of concurrent agonist administration. *Brain Res. Bull.* **1994**, *33*, 237-240.
(45) Nwokolo, C. U.; Smith, J. T.; Sawyerr, A. M.; Pounder, R. E. Rebound intragastric hyperacidity after abrupt withdrawal of histamine H2 receptor blockade. *Gut* **1991**, *32*, 1455-1460.
(46) Merki, H. S.; Wilder-Smith, C. H. Do continuous infusions of omeprazole and ranitidine retain their effect with prolonged dosing? *Gastroenterology* **1994**, *106*, 60-64.
(47) Ngochindo, R. I. 2-Protecting Groups for 5-Lithiation in the Syntheses of Imidazoles. *J. Chem. Soc., Perkin Trans. 1* **1990**, *6*, 1645-1648.

(48) Mitsunobu, O. The Use of Diethyl Azodicarboxylate and Triphenylphosphine in Synthesis and Transformation of Natural Products. *Synthesis* **1981**, 1-28.
(49) Gibson, M. S.; Bradshaw, R. W. Gabriel-Synthese Primärer Amine. *Angew. Chem.* **1968**, *80*, 986-996.
(50) Horne, D. A.; Yakushijin, K.; Büchi, G. A Two-Step Synthesis of Imidazoles From Aldehydes Via 4-Tosyloxazolines. *Heterocycles* **1994**, *39*, 139-153.
(51) Loudon, G. M.; Radhakrishna, A. S.; Almond, M. R.; Blodgett, J. K.; Boutin, R. H. Conversion of Aliphatic Amides into Amines with [*I,I*-Bis(trifluoroacetoxy)iodo]benzene. 1. Scope of the Reaction. *J. Org. Chem.* **1984**, *49*, 4272-4276.
(52) Brandsma, L. Metallation of Heterocyclic Compounds (Chapter 6). *Preparative Polar Organometallic Chemistry*; Springer-Verlag: Berlin, 1990; pp 127-139.
(53) Vollinga, R. C.; Menge, W. M.; Leurs, R.; Timmerman, H. Homologs of histamine as histamine H_3 receptor antagonists: a new potent and selective H_3 antagonist, 4(5)-(5-aminopentyl)-1H-imidazole. *J. Med. Chem.* **1995**, *38*, 266-271.
(54) Walter, L. A.; Hunt, W. H.; Fosbinder, R. J. *J. Am. Chem. Soc.* **1941**, *63*, 2771.
(55) Zingel, V.; Leschke, C.; Schunack, W. Developments in histamine H_1-receptor agonists. *Prog. Drug Res.* **1995**, *44*, 49-85.
(56) Leschke, C.; Elz, S.; Garbarg, M.; Schunack, W. Synthesis and histamine H1 receptor agonist activity of a series of 2- phenylhistamines, 2-heteroarylhistamines, and analogues. *J. Med. Chem.* **1995**, *38*, 1287-1294.
(57) ter Laak, A. M.; Timmerman, H.; Leurs, H.; Nederkoorn, P. H. J.; Smit, M. J.; Donne-Op den Kelder, G. M. Modelling and mutation studies of the histamine H_1 receptor agonist binding site reveal different binding modes for H_1 agonists: Asp^{116} (TM3) has a constitutive role in receptor stimulation. *J. Comp. Aid. Mol. Design.* **1995**, *9*, 319-330.
(58) Elz, S.; Kramer, K.; Pertz, H. H.; Detert, H.; ter Laak, A. M.; Kühne, R.; Schunack, W. Histaprodifens: synthesis, pharmacological in vitro evaluation, and molecular modeling of a new class of highly active and selective histamine H_1-receptor agonists. *J. Med. Chem.* **2000**, *43*, 1071-1084.
(59) Elz, S.; Kramer, K.; Leschke, C.; Schunack, W. Ring-substituted histaprodifen analogues as partial agonists for histamine H_1 receptors: synthesis and structure-activity relationships. *Eur. J. Med. Chem.* **2000**, *35*, 41-52.
(60) Walczynski, K.; Timmerman, H.; Zuiderveld, O. P.; Zhang, M. Q.; Glinka, R. Histamine H_1 receptor ligands. Part I. Novel thiazol-4-ylethanamine derivatives: synthesis and in vitro pharmacology. *Farmaco* **1999**, *54*, 533-541.
(61) Walczynski, K.; Guryn, R.; Zuiderveld, O. P.; Zhang, M. Q.; Timmerman, H. Histamine H_1 receptor ligands: part II. Synthesis and in vitro pharmacology of 2-[2-(phenylamino)thiazol-4-yl]ethanamine and 2-(2- benzhydrylthiazol-4-yl)ethanamine derivatives. *Farmaco* **2000**, *55*, 569-574.
(62) Ter Laak, A. M.; Venhorst, J.; Donné-Op den Kelder, G. A.; Timmerman, H. The Histamine H_1-Receptor Antagonist Binding Site. A Stereoselective Pharmacophoric Model Based upon (Semi-)Rigid H_1-Antagonists and Including a Known Interaction Site on the Receptor. *J. Med. Chem.* **1995**, *38*, 3351-3360.
(63) Elz, S.; Kramer, K.; Leschke, C.; Schunack, W. Ring-substituted Histaprodifens Analogues as Partial Agonists for Histamine H_1 Receptor: Synthesis and Structure-activity Relationships. *Eur. J. Med. Chem.* **2000**, *35*, 41-52.
(64) Berg, K. A.; Stout, B. D.; Cropper, J. D.; Maayani, S.; Clarke, W. P. Novel actions of inverse agonists on 5-HT_{2C} receptor systems. *Mol. Pharmacol.* **1999**, *55*, 863-872.
(65) Lavreysen, H.; Le Poul, E.; Van Gompel, P.; Dillen, L.; Leysen, J. E.; Lesage, A. S. J. Supersensitivity of Human Metabotropic Glutamate 1a Receptor Signaling in L929sA Cells. *Mol. Pharmacol.* **2002**, *61*, 1244-1254.
(66) Wallace, R. M.; Young, J. M. Temperature dependence of the binding of [^{3}H]mepyramine and related compounds to the histamine H_1 receptor. *Mol. Pharmacol.* **1983**, *23*, 60-66.
(67) Wieland, K.; ter Laak, A. M.; Smit, M. J.; Kühne, R.; Timmerman, H.; Leurs, R. Mutational analysis of the antagonist-binding site of the histamine H_1 receptor. *J. Biol. Chem.* **1999**, *274*, 29994-30000.

(68) Ahmad, K.; Strong, F. M. The Synthesis of Unsaturated Fatty Acids. *J. Am. Chem. Soc.* **1948**, *70*, 1699-1700.
(69) Carbelleira, N.; Thompson, J. E.; Ayanoglu, E.; Djerassi, C. Biosynthetic Studies of Marine Lipids. 5. The Biosynthesis of Long-Chain Branched Fatty Acids in Marine Sponges. *J. Org. Chem.* **1986**, *51*, 2751-2756.
(70) Heiser, U. F.; Dobner, B. Copper-catalysed Coupling of Undec-10-enylmagnesium Bromide with w-Functionalised Halogenoalkanes as a Key Reaction for the Synthesis of Novel Bipolar Phospholipids with Different Head Groups and Chain Length. *J. Chem. Soc., Perkin Trans. 1* **1997**, *1*, 805-815.
(71) Cundy, D. J.; Gurr, P. A. An Improved Synthesis of +/- 18-methyl-eicosanoic acid. *Org. Prep. Proced. Int.* **2000**, *32*, 461-468.
(72) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254.

Chapter 4

A chemical switch for the modulation of the functional activity of higher homologues of histamine on the human histamine H_3 receptor: effect of various substitutions at the primary amino function

Marinella Govoni, Herman D. Lim, Dris El-Atmioui, Wiro M.P.B. Menge, Henk Timmerman, Remko A. Bakker, and Rob Leurs

Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Abstract

In an effort to establish the structural requirements for agonism, neutral antagonism and inverse agonism at the human histamine H_3 receptor (H_3R) we have prepared a series of higher homologues of histamine in which the terminal nitrogen of the side chain has been either mono- or di-substituted with several aliphatic, alicyclic and aromatic moieties, or incorporated in cyclic systems. The novel ligands have been pharmacologically investigated *in vitro* for their affinities on the human H_3R and H_4R subtypes by radioligand displacement experiments, and for their intrinsic H_3R activities via a CRE-mediated β-galactosidase reporter-gene assay. Subtle changes of the substitution pattern at the side chain nitrogen alter enormously the pharmacological activity of the ligands, resulting in a series of compounds with a wide spectrum of pharmacological activities. Among the several neutral H_3R antagonists identified within this series, compounds **2b** and **2h** display an H_3R affinity in the low nanomolar concentration range (pK_i values of 8.1 and 8.4, respectively). A very potent and selective H_3R agonist (**1m**, pEC_{50}=8.9, α=0.94) and a very potent, though not highly selective, H_3R inverse agonist (**2l**, pIC_{50}=8.9, α=-0.97) have been identified as well.

Introduction

The biogenic amine histamine exerts its multiple biological activities through the activation of at least four distinct histamine receptors, i.e., H_1, H_2, H_3 and H_4 receptors,[1-4] all belonging to the superfamily of heptahelical (7TM) G-protein coupled receptors.

The histamine H_3 receptor (H_3R) was discovered in 1983 by Arrang and coworkers as a presynaptic autoreceptor,[5] and the gene successively cloned in 1999 by Lovenberg and colleagues.[3] Tissue distribution analysis indicated that the expression of the receptor is predominantly restricted to the brain.[6] The H_3R mediates the inhibition of synthesis and release of histamine from histaminergic neurons via a negative feedback loop[7,8] but also exerts modulatory effects on other neurotransmitter systems, e.g. the cholinergic,[9,10] dopaminergic,[11] noradrenergic[12] and serotoninergic[13] systems, in both the central and peripheral nervous systems. It has been recognized that the H_3R is a potential therapeutic target.[14-16] H_3R antagonists have been proposed to be potential drugs for the treatment of several CNS disorders, such as attention-deficit hyperactivity disorder (ADHD),[17,18] Alzheimer's disease,[19] epilepsy,[20-22] schizophrenia[22,23] and obesity,[24-26] whereas the therapeutic potential of H_3R agonists has been shown for myocardial ischemia,[27] inflammatory[28] and gastric acid related diseases,[29] migraine and sleep disorders.[30]

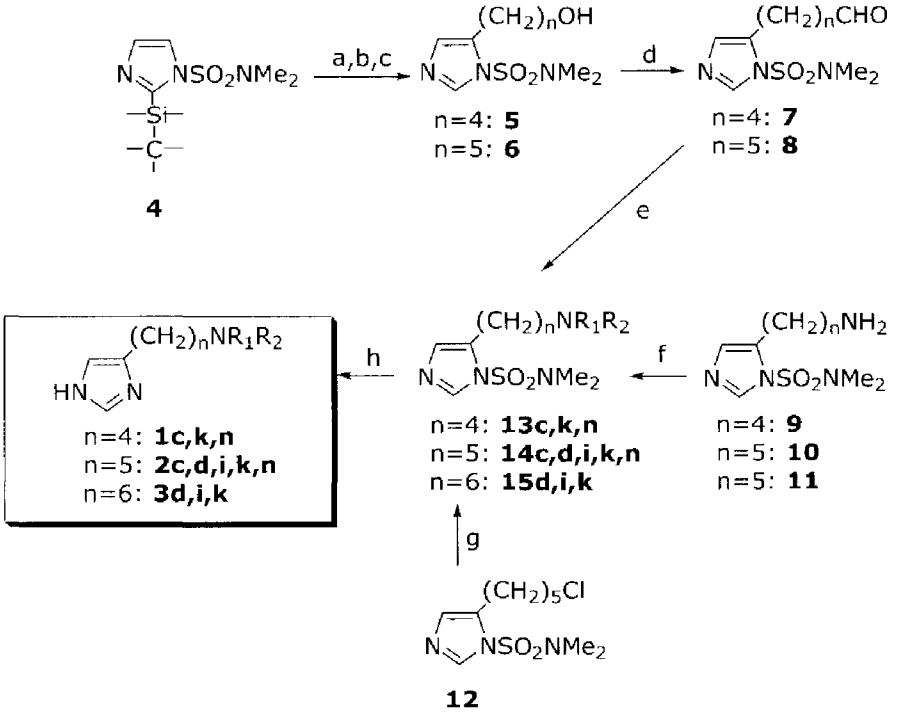
Constitutive activity of several 7TM receptor systems has been investigated within the past decade.[31-36] For a long time it was not known whether this phenomenon existed only in cells, overexpressing (mutant) GPCRs or also occurred *in vivo*. Constitutive activity has been shown recently for both the histamine H_1 and H_2 receptors.[37,38] Our group reported that the therapeutically important H_1 and H_2 receptor antagonists act, in fact, as inverse agonists, but we also identified neutral antagonists for both receptors.[37,39] Histaminergic neurotransmission in rodent brain was shown to be regulated by constitutively active H_3Rs *in vitro* as well as *in vivo*,[40] and our group also demonstrated that the human and rat H_3Rs stably expressed in SK-N-MC cells[3,6] show a high level of constitutive activity.[41,42] This led to the identification of several standard H_3R antagonists (thioperamide and clobenpropit) as inverse agonists in this cell system. Moreover, burimamide and impentamine (Figure 1), previously identified as H_3R antagonists,[5,43,44] behave as H_3R agonists at both recombinant H_3Rs (human and rat). We also showed that in a small series of impentamine analogues we were able to manipulate the intrinsic activity (α) by substitution of the amino group.[41]

In an attempt to establish a correlation between the structure of the ligands and their functional activity at the human H_3R, we report here the synthesis, affinity and intrinsic H_3R activity of a variety of analogues of imbutamine (**1a**), impentamine (**2a**) and imhexamine (**3a**, Figure 1), in which the primary amino function has been mono- or di-substituted with several aliphatic and aromatic moieties. All the ligands were additionally screened for their affinities at

the human histamine H_4 receptor (H_4R) subtype in order to evaluate H_3 versus H_4 receptor selectivity of the compounds.

Chemistry

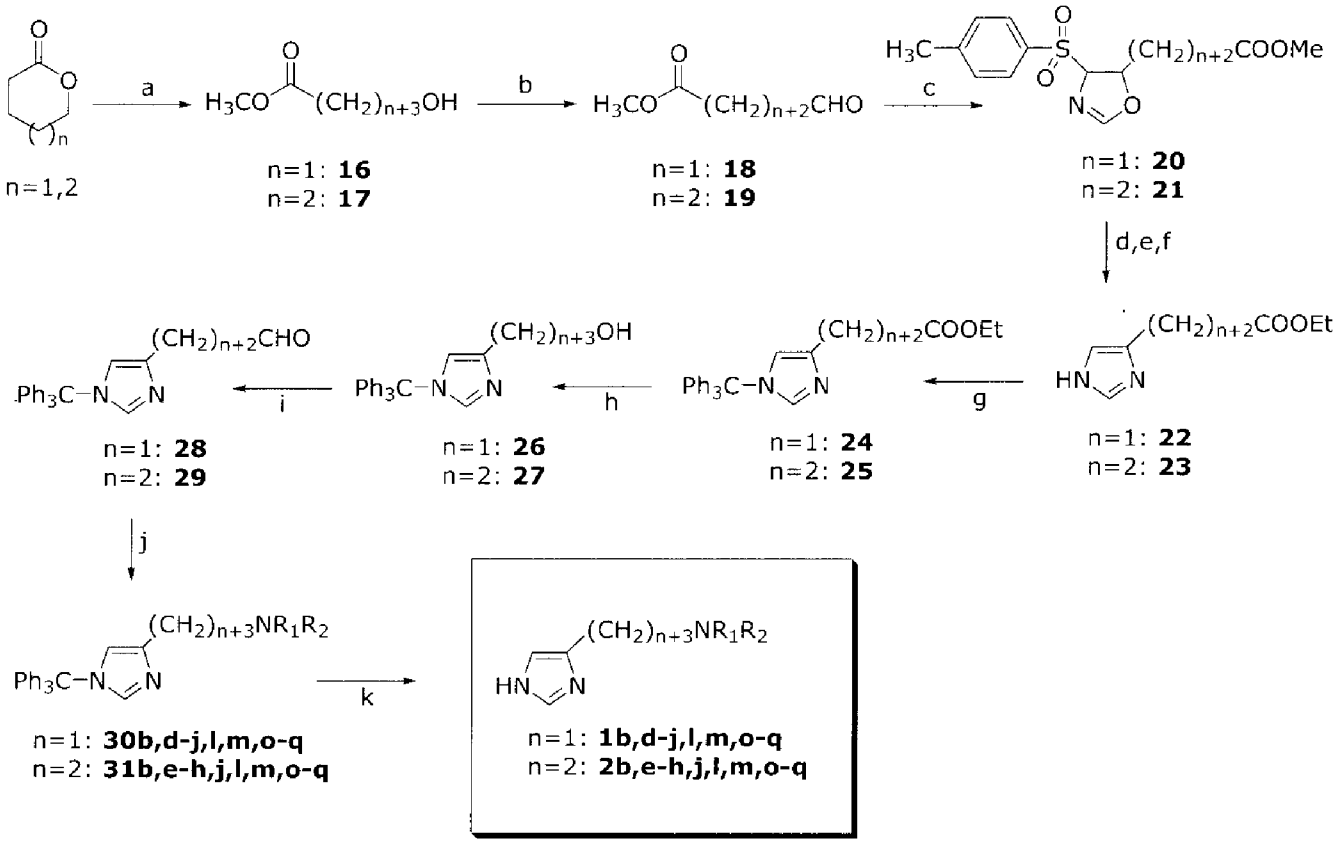
Aminoalkylimidazoles **1c,n** and **2c,n** were prepared by lithiation of a suitable 1,2-diprotected imidazole **4**[45] and subsequent treatment with 2-(ω-iodo-alkyloxy)-tetrahydro-pyrans to give compounds **5** and **6** in moderate yield (35-40%, Scheme 1).



Scheme 1. Synthesis of compounds **1c,k,n**, **2c,d,i,k,n** and **3d,i,k,**. Reagents: (a) *n*-BuLi, THF, -70°C; (b) 2-(ω-iodo-alkyloxy)-tetrahydro-pyran; (c) HCl, r.t.; (d) Swern oxidation; (e) amine, AcOH, $NaBH(OAc)_3$, DCE, r.t. (Method C, reductive amination); (f) aldehyde or ketone, cat. PtO_2, EtOH, H_2, 50 bar (Method A); (g) amine, 100 °C (Method B); (h) 30% HBr or 37% HCl, reflux (Method E).

The ω-hydroxy group of **5** and **6** was converted into an aldehyde via a Swern oxidation[46,47] followed by reductive amination[48] with the appropriate amine in the presence of sodium triacetoxyborohydride and cleavage of the dimethylsulfamoyl protecting group to give compounds **1c,n** and **2c,n** in moderate to good yield (44-70%). Alternatively, the ω-chloro group of **12**[44] was converted either into an isopropylamino group or into a cyclohexylamino group via heating with isopropylamine and cyclohexylamine, respectively, to give compounds **14d,i**. Deprotection yielded compounds **2d,i**. Compounds **1k**, **2k** and **3d,i,k** were synthesized starting from the protected higher homologues of histamine **9**, **10** and **11** and benzaldehyde (**13k**, **14k**, **15k**), acetone (**15d**) or cyclohexanone (**15i**) via reduction with H_2 (50 bar) in the presence of a catalytic amount of PtO_2. Removal of the dimethylsulfamoyl protecting group afforded the final compounds **1k**, **2k** and **3d,i,k** (26-54% yield).

Aminoalkylimidazoles **1b,d-j,l,m,o-q** and **2b,e-h,j,l,m,o-q** were prepared according to the synthetic pathway shown in Scheme 2.



Scheme 2. Synthesis of compounds **1b,d-j,l,m,o-q** and **2b,e-h,j,l,m,o-q**. Reagents: (a) MeOH, H_2SO_4, reflux; (b) Swern oxidation; (c) TosMIC, NaCN, EtOH abs.; (d) NH_3/EtOH, 120-140°C, 10 atm; (e) HCl, reflux; (f) EtOH, H_2SO_4, reflux; (g) triphenylmethyl chloride, triethylamine, DCM, r.t.; (h) $LiAlH_4$, THF, r.t.; (i) Swern oxidation; (j) amine, AcOH, $NaBH(OAc)_3$, DCE, r.t. (Method C, reductive amination); (k) HCl 1M or HBr 1M, reflux (Method D).

Methanolysis of δ-valerolactone and ε-caprolactone followed by Swern oxidation of esters **16** and **17** afforded aldehydes **18** and **19**, which were readily converted into imidazoles using TosMIC chemistry.[49] The intermediate 4-tosyloxazolines **20** and **21** were treated with ammonia in ethanol under pressure affording a mixture of amide, methyl and ethyl ester which were converted into the carboxylic acid via acidic hydrolysis and then re-esterified to give esters **22** and **23** in good yield (82% and 51%, respectively). Protection of the imidazole ring with a trityl group, followed by reduction of esters **24** and **25** with lithium aluminumhydride and Swern oxidation of the alcohols **26** and **27** yielded the key intermediates **28** and **29** (49% and 70% yield). Reductive amination with a suitable amine and subsequent deprotection afforded the final compounds **1b,d-j,l,m,o-q** and **2b,e-h,j,l,m,o-q**, in variable yield (17-95%).

Pharmacology

Radioligand Displacement Studies. Homogenates of SK-N-MC cells, stably expressing either the human H_3R[3] or the human H_4R,[50] were used for determining ligand affinities for the H_3R and H_4R respectively, as previously described.[51] Cell homogenates of H_3R-expressing cells (475 ± 32 fmol/mg of protein) were incubated for 40 min at 25 °C with 0.9-1.1 nM [^{3}H]-N^α-methylhistamine (82 Ci/mmol) in 50 mM sodium phosphate buffer (pH 7.4) with or without competing ligands; cell homogenates of H_4R-expressing cells (620 ± 44 fmol/mg of protein) were incubated for 60 min at 37 °C with 9-11 nM [^{3}H]histamine (23.2 Ci/mmol) in 50 mM Tris HCl (pH 7.4), with or without competing ligands. Incubations were terminated by rapid dilution and subsequent filtration over Whatman GF/C filters pretreated with 0.3% polyethyleneimine using ice-cold wash buffer (H_3R: 25mM Tris HCl, 145 mM NaCl, pH 7.4 at 4 °C; H_4R: 50mM Tris HCl, pH 7.4 at 4 °C). The radioactivity retained on the filters was measured by liquid scintillation counting. Nonspecific binding was determined with 1 μM thioperamide as competing ligand. Competition isotherms were evaluated by a nonlinear, least squares curve-fitting using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Colorimetric cAMP Assay. Colorimetric cAMP assays were performed as previously described.[51] Briefly, SK-N-MC cells stably expressing the human H_3R[3] and a cyclic AMP responsive element (CRE)-β-galactosidase reporter-gene were grown overnight in 96-well plates before the assay. To start the assay, the cells were incubated for 6 h with 1 μM forskolin and respective ligands at 37 °C. Thereafter, the medium was aspirated and cells were incubated overnight at 4 °C with 100 μL of assay buffer [100 mM NaH_2PO_4, 100 mM Na_2HPO_4, pH 8, 2 mM $MgSO_4$, 0.1 mM $MnCl_2$, 0.5% Triton, 40 mM β-mercaptoethanol, and 4 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG)]. The absorbance at 405 nm was determined by using a Victor^2 plate reader (Perkin-Elmer).

Analytical Methods. Protein levels were determined spectrophotometrically by a Packard Argus 400 microplate reader according to the method of Bradford,[52] using bovine serum albumin as a standard. All data shown are expressed as a mean ± S.E.M., and statistical analyses were carried out by Student's t-test. P values < 0.05 were considered to indicate a significative difference.

Results and discussion

A previous study from our group identified compound VUF 4904 (**2d**, Figure 1 and Table 2), an impentamine analogue with an isopropyl group at the amino group of the side chain, as a neutral H_3R antagonist[41] in SK-N-MC cells stably transfected with the human H_3R.[3]

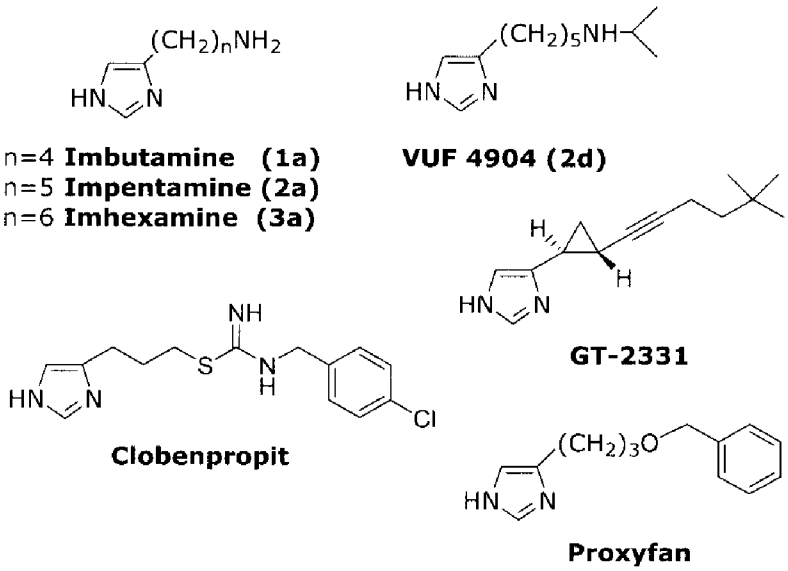


Figure 1. Structures of 4-[1*H*-imidazol-4-yl]-butylamine (imbutamine, **1a**), 5-[1*H*-imidazol-4-yl]-pentylamine (impentamine, **2a**), 6-[1*H*-imidazol-4-yl]-hexylamine (imhexamine, **3a**), [5-(1*H*-imidazol-4-yl)-pentyl]-isopropyl-amine (VUF 4904, **2d**), clobenpropit, GT-2331 (cipralisant, Perceptin®) and proxifan.

In the same study it was also shown that the modification of the primary amino group of the H_3R agonist impentamine, via substitution or incorporation in a piperidine ring, dramatically affected the pharmacological activity of the ligands, resulting in a series of compounds spanning the whole spectrum of pharmacological activities.

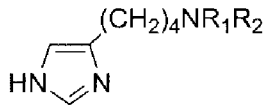
The amine function of impentamine is thought to interact with the aspartate residue Asp^{114} in transmembrane domain 3, which is highly conserved in the family of biogenic amine receptors.[53,54]

In view of the fact that this series of ligands may be of great help to understand the mechanism of receptor (in)activation, we have prepared a series of imbutamine and impentamine analogues in which the primary amino function has been mono- or di-substituted with either a variety of aliphatic (**1b-g,m,n**, **2b-g,m,n**), alicyclic (**1h-j**, **2h-j**) and aromatic moieties (**1k,l**, **2k,l**), or incorporated in cyclic systems (**1o-q**, **2o-q**). Some of these modifications of the side chain terminal nitrogen were applied to imhexamine (**3a**, Figure 1) as well, leading to analogues **3d,i,k** (Table 3).

All the synthesized compounds have been investigated for the effects caused by the modification on the amine moiety of the side chain on the human H_3R affinity, potency and intrinsic activity. The functional activity was determined by the inhibition or activation of the forskolin-stimulated (1μM) cAMP production in SK-N-MC cells stably expressing the human

H_3R,[3] via a CRE-driven β-galactoxidase reporter-gene assay. As shown in Table 1, substitution of the N^{α} position of imbutamine (**1a**) caused in general a decrease in the H_3R affinity, with the exception of compounds **1f**, bearing an *i*-butyl substituent, and N^{α}-N^{α}-dimethylimbutamine (**1m**), in which the terminal amino function is dimethylated.

Table 1. Chemical structures and pharmacological properties of compounds **1a-q** for the human H_3 (hH_3R) and H_4 (hH_4R) receptors. The values are expressed as means ± SEM of separate experiments, each performed in triplicate, unless indicated otherwise.



				hH_3R			hH_4R
compd	name or code	R_1	R_2	pK_i[a]	pEC_{50}[a]	α[a,b]	pK_i
1a	imbutamine	H	H	8.4 ± 0.1	8.3 ± 0.1	0.98 ± 0.02	8.0 ± 0.1
1b	VUF 5847	H	Et	7.6 ± 0.1	7.6 ± 0.1	0.73 ± 0.10	7.0 ± 0.1
1c	VUF 5737	H	nPr	8.1 ± 0.1	7.8 ± 0.1	0.45 ± 0.07	7.3 ± 0.1
1d	VUF 5827	H	iPr	7.1 ± 0.1	-[c]	0.10 ± 0.08	6.7 ± 0.1
1e	VUF 5844	H	nBu	8.2 ± 0.1	8.3 ± 0.1	0.55 ± 0.09	7.2 ± 0.1
1f	VUF 5738	H	iBu	8.5 ± 0.1	8.1 ± 0.1[d]	-0.14 ± 0.10	7.1 ± 0.1
1g	VUF 5850	H	tBu	6.2 ± 0.1	-[c]	0.01 ± 0.06	6.1 ± 0.1
1h	VUF 5852	H	cyclopentyl	7.4 ± 0.1	-[e]	-[c]	7.4 ± 0.1
1i	VUF 5828	H	cyclohexyl	7.5 ± 0.1	-[e]	-[c]	7.0 ± 0.1
1j	VUF 5854	H	cycloheptyl	7.4 ± 0.1	-[c]	0.00 ± 0.05	7.3 ± 0.0
1k	VUF 4807	H	benzyl	7.3 ± 0.1	7.4 ± 0.1	0.72 ± 0.08	7.1 ± 0.1
1l	VUF 5829		*p*-Cl-benzyl	7.8 ± 0.1	-[e]	-[c]	7.7 ± 0.1
1m	VUF 5826	Me	Me	8.5 ± 0.1	8.9 ± 0.1	0.94 ± 0.04	6.1 ± 0.1
1n	VUF 5739	Et	Et	7.0 ± 0.1	-[c]	0.07 ± 0.08	6.5 ± 0.1
1o	VUF 5845	-$(CH_2)_4$-		7.4 ± 0.1	7.4 ± 0.1	0.47 ± 0.11	6.3 ± 0.1
1p	VUF 5825	-$(CH_2)_5$-		7.7 ± 0.1	7.5 ± 0.1	0.64 ± 0.04	6.4 ± 0.2
1q	VUF 5843	-$(CH_2)_6$-		7.4 ± 0.1	7.3 ± 0.2	0.35 ± 0.07	6.3 ± 0.1

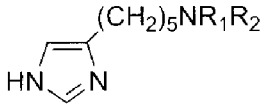
[a] N>3. [b] α=intrinsic activity value. [c] Could not be estimated. [d] pIC_{50} value. [e] biphasic behaviour (partial agonism up to 1 μM, non-H_3R-mediated effetcs at 10 and 100 μM).

With a pEC_{50} of 8.9, **1m** is the most potent ligand of this series and it displays a full agonistic intrinsic activity (α=0.94) on the human H_3R. This behaviour has already been observed in the case of histamine, where mono- or dimethylation of the amino function of the side chain results in agonists that are more active than histamine at the H_3R.[5,55] Surprisingly, the dimethylated analogue of impentamine, **2m** (Table 2), shows instead reduced affinity and potency, with a pK_i value of 7.8 and a pEC_{50} value of 7.5, as well as a diminished intrinsic H_3R activity, resulting in partial H_3R agonism (α=0.68). It must be noted, however, that the parent compound **2a**, opposite to what was observed in our previous study where it behaved as a full agonist (α=0.9),[41] is now found to be also a partial agonist (α=0.71), with almost the same intrinsic activity as **2m**. Further increase of the steric hindrance on the primary amine, as achieved via introduction of a diethylamino group, is generally not well tolerated, as shown by the decreased H_3R affinities of compounds **1n** and **2n**. Parallel to the decrease in the H_3R

affinity a diminished H_3R intrinsic activity is observed, in fact both ligands are neutral H_3R antagonists.

In the impentamine series shown in Table 2, compounds **2c**, **2e**, **2f**, **2h**, **2j** and **2l** display an H_3R affinity comparable to the parent compound **2a** or slightly increased.

Table 2. Chemical structures and pharmacological properties of compounds **2a-q** for the human H_3 (hH_3R) and H_4 (hH_4R) receptors. The values are expressed as means ± SEM of separate experiments, each performed in triplicate, unless indicated otherwise.



				hH_3R			hH_4R
compd	name or code	R_1	R_2	pK_i[a]	pEC_{50}[a]	α[a,b]	pK_i
2a	impentamine	H	H	8.3 ± 0.1	8.3 ± 0.1	0.71 ± 0.05	6.6 ± 0.1
2b	VUF 5848	H	Et	8.1 ± 0.1	-[c]	0.11 ± 0.09	6.5 ± 0.1
2c	VUF 5740	H	nPr	8.3 ± 0.1	8.0 ± 0.1	0.44 ± 0.11	6.8 ± 0.1
2d	VUF 4904	H	iPr	7.9 ± 0.1	7.4 ± 0.1[d]	-0.55 ± 0.11	6.9 ± 0.1
2e	VUF 5849	H	nBu	8.4 ± 0.1	8.3 ± 0.1	0.55 ± 0.09	6.7 ± 0.1
2f	VUF 5842	H	iBu	8.8 ± 0.1	8.1 ± 0.2	0.35 ± 0.07	6.9 ± 0.1
2g	VUF 5851	H	tBu	7.4 ± 0.1	-[c]	0.04 ± 0.05	7.2 ± 0.1
2h	VUF 5853	H	cyclopentyl	8.4 ± 0.1	-[c]	0.02 ± 0.08	7.4 ± 0.1
2i	VUF 4903	H	cyclohexyl	8.0 ± 0.1	7.9 ± 0.1[d]	-0.51 ± 0.12	7.4 ± 0.1
2j	VUF 5855	H	cycloheptyl	8.5 ± 0.1	8.0 ± 0.1[d]	-0.65 ± 0.03	7.3 ± 0.1
2k	VUF 4808	H	benzyl	8.0 ± 0.1	7.8 ± 0.1[d]	-0.56 ± 0.04	6.6 ± 0.0
2l	VUF 5202		*p*-Cl-benzyl	8.6 ± 0.1	8.9 ± 0.2[d]	-0.97 ± 0.01	7.2 ± 0.2
2m	VUF 5207	Me	Me	7.8 ± 0.2	7.5 ± 0.2	0.68 ± 0.08	6.3 ± 0.1
2n	VUF 5741	Et	Et	7.2 ± 0.1	-[c]	0.06 ± 0.02	6.2 ± 0.1
2o	VUF 5846		$-(CH_2)_4-$	7.5 ± 0.1	7.5 ± 0.1	0.32 ± 0.07	6.1 ± 0.1
2p	VUF 5300		$-(CH_2)_5-$	8.1 ± 0.1	7.7 ± 0.1	0.46 ± 0.08	6.5 ± 0.1
2q	VUF 5856		$-(CH_2)_6-$	7.8 ± 0.1	7.3 ± 0.2	0.22 ± 0.07	6.8 ± 0.1

[a] N>3. [b] α=intrinsic activity value. [c] Could not be estimated. [d] pIC_{50} value.

Comparing imbutamine and impentamine derivatives it can be appreciated that, with the exception of the parent compounds (**1a**, **2a**) and those bearing a dimethyl substitution (**1m**, **2m**), the H_3R affinities are higher for the impentamine analogues. Further elongation of the side chain does not improve the H_3R affinity or the potency. In fact, imhexamine (**3a**, Table 3) shows a decreased H_3R affinity and potency, and the same is true for all of its analogues, except **3i**, bearing a cyclohexyl residue. Alkylation in the N^{α}-position of **1a** and **2a** with longer alkyl groups, such as ethyl, *n*-propyl or *n*-butyl results in an increase in the H_3R affinity in both series (**1b**,**c**,**e** and **2b**,**c**,**e**), whilst ramification to *i*-propyl (**1d**, **2d**) or *t*-butyl (**1g**, **2g**) is detrimental for H_3R affinity (see Tables 1 and 2). Alkylation of the histamine side-chain terminal nitrogen with bulky groups leads to a loss of H_3R intrinsic activity, generating partial agonists[56] or antagonists.[57] Similarly, alkylation of imbutamine and impentamine with the above mentioned residues, e.g. ethyl, *n*-propyl, *i*-propyl, *n*-butyl and *t*-butyl causes a decrease in the intrinsic activity of the compounds, therefore generating partial agonists (**1b**,**c**,**e** and

2c,e) or neutral antagonists (**1d,g** and **2b,g**, Tables 1,2). The *i*-propyl moiety is conferring neutral H_3R antagonism to the imhexamine derivative **3d** as well (Table 3). Remarkably, compound **2d** (Table 2), also bearing an *i*-propyl moiety, that was identified as a neutral antagonist (α=-0.10 ± 0.10) in our previous study,[41] in the present research behaves as a partial inverse agonist (α=-0.55).

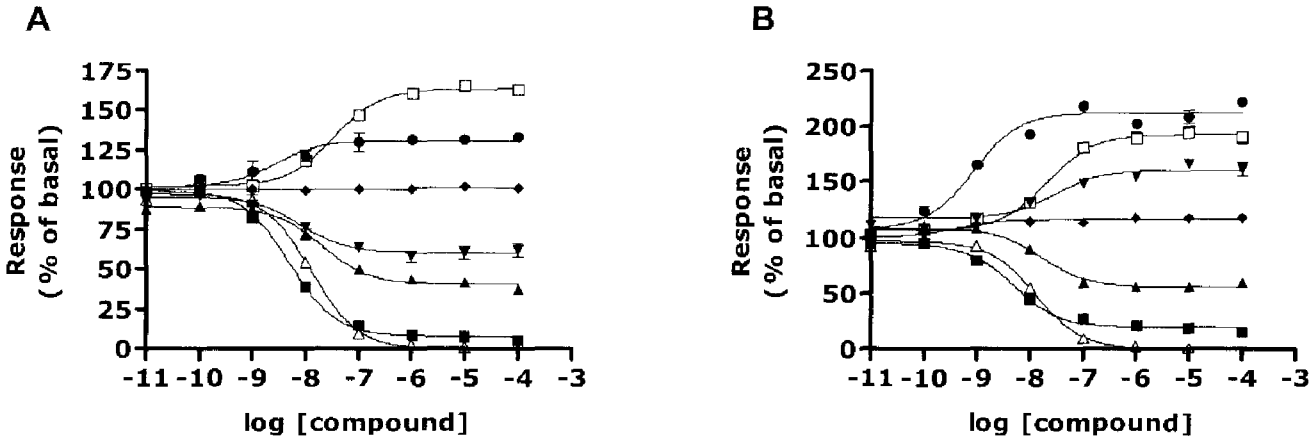


Figure 2. The effects of several selected compounds on the basal signalling of the H_3R, as measured by CRE-mediated β-galactosidase reporter gene assay in forskolin-stimulated (1μM) SK-N-MC cells. A, Dose-response curves of the full H_3R agonist VUF 4701 (■, imbutamine, **1a**), the partial H_3R agonists VUF 5847 (▲, **1b**) and VUF 5737 (▼, **1c**), the neutral H_3R antagonist VUF 5850 (♦, **1g**) and the partial inverse H_3R agonists VUF 5738 (●, **1f**) (see also Table 1). B, Dose-response curves of the partial H_3R agonists VUF 4702 (■, impentamine, **2a**) and VUF 5740 (▲, **2c**), the neutral H_3R antagonist VUF 5853 (♦, **2h**), the partial inverse H_3R agonist VUF 4904 (▼, **2d**) and the full inverse H_3R agonist VUF 5202 (●, **2l**) (see also Table 2). Representative dose-response curves of the full H_3R agonist histamine (△) and of the partial inverse H_3R agonist thioperamide (□) are shown (A and B). Data are normalised to the basal signalling observed in the assay (set to 100%).

However, a different assay system has been used in the current study, which is more sensitive than the classical cAMP measurements used in the previous study.[41] Thus, enhancement of the Van der Waals volume of the N^{α}-position diminishes agonist properties and increases antagonistic activity, but no definite relation between the substituting alkyl residue and the intrinsic H_3R activity of the compound can be derived. Surprisingly, ramification to *i*-butyl (**1f** and **2f**) generates compounds with higher H_3R affinity, in fact compound **2f** (Table 2), with a pK_i of 8.8, exhibits the highest H_3R affinity among this series of ligands. Unfortunately, no definite correlation between the alkyl residue and the intrinsic H_3R activity could be observed. In fact, **1f** shows partial inverse agonism (α=-0.14, Table 1), whereas **2f** is a partial agonist (α=0.35, Table 2).

Substitution with alicyclic residues (cyclopentyl, cyclohexyl or cycloheptyl) exerts a different behaviour for imbutamine and impentamine derivatives; in the former, alkylation with either of the moieties produces a ten-fold decrease in the H_3R affinity (**1h-j**, Table 1), whereas in the

latter H_3R affinities are not significantly altered (**2h-j**, Table 2). As already seen in case of alkylation with aliphatic residues, insertion of alicyclic residues results in a loss of agonistic activity compared to the unsubstituted compounds; in fact, **1j** and **2h** are neutral antagonists, whereas **2i** and **2j** are partial inverse agonists.

In view of its nanomolar affinity for the H_3R (pK_i=8.4) and lack of intrinsic H_3R activity (α=0.02 ± 0.08), N^{α}-cyclopentylimpentamine (**2h**, Table 2) could be a novel potentially useful pharmacological tool as well as a novel lead. As depicted in Figure 3, ligand **2h** effectively antagonizes the response to the full agonist imbutamine (**2a**, Figure 3) yielding a pA_2 value of 8.1.

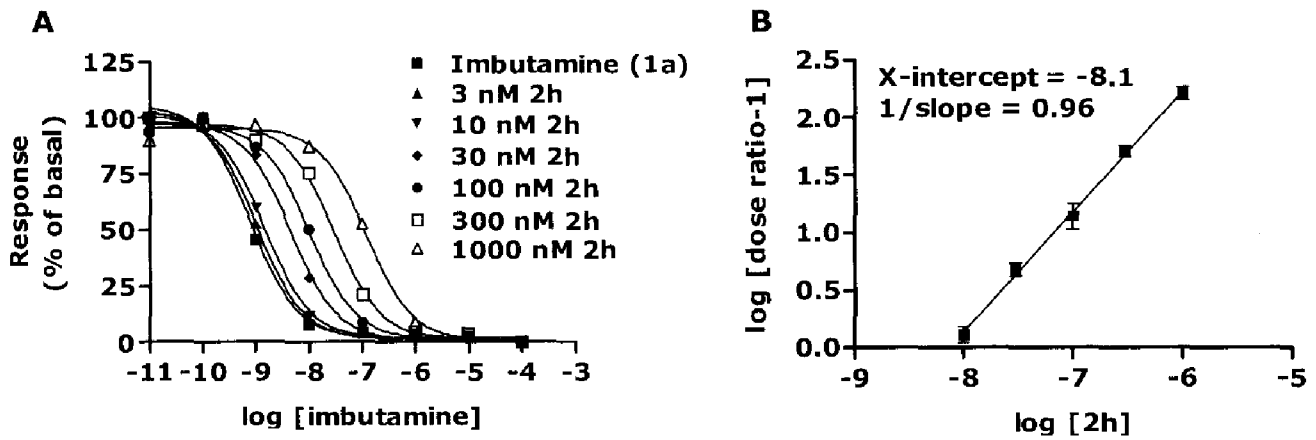
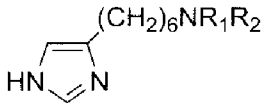


Figure 3. A, Rightward shift of concentration-response curves of imbutamine (**1a**) in the presence of increasing concentrations of N^{α}-cyclopentylimpentamine (**2h**) determined by the inhibition of the CRE-stimulated β-galactosidase transcription in SK-N-MC cells stably expressing the human H_3R. B, The log of the (dose ratio–1) at each concentration of antagonist was plotted versus the log of **2h** concentration. The pA_2 value was determined from the *x*-intercept on the linear Schild regression plot and was in this particular experiment 8.1. The data points represent means ± S.E.M. of triplicate determinations from one representative experiment.

Insertion of a cyclohexyl group on imhexamine also results in a reduced intrinsic activity, shifting it from partial agonism of **3a** (α=0.25) to inverse agonism of **3i** (α=-0.63) (Table 3).

Table 3. Chemical structures and pharmacological properties of compounds **3a,d,i,k** for the human H_3 (hH_3R) and H_4 (hH_4R) receptors. The values are expressed as means ± SEM of separate experiments, each performed in triplicate, unless indicated otherwise.



				hH_3R			hH_4R
compd	name or code	R_1	R_2	pK_i[a]	pEC_{50}[a]	α[a,b]	pK_i
3a	imhexamine	H	H	7.6 ± 0.1	7.7 ± 0.1	0.25 ± 0.09	6.2 ± 0.1
3d	VUF 4852	H	iPr	7.2 ± 0.1	-[c]	-0.18 ± 0.14	6.4 ± 0.1
3i	VUF 4810	H	cyclohexyl	8.3 ± 0.1	7.9 ± 0.1[d]	-0.63 ± 0.07	7.3 ± 0.1
3k	VUF 4809	H	benzyl	7.6 ± 0.1	7.7 ± 0.1[d]	-0.63 ± 0.07	6.9 ± 0.1

[a] N>3. [b] α=intrinsic activity value. [c] Could not be estimated. [d] pIC_{50} value.

Noticeably, the H_3R affinity of **3i** was higher than the one of the parent compound (pk_i=8.3 vs 7.6 of **3a**, Table 3). Ligands **1h** and **1i** (Table 1), respectively alkylated with a cyclopentyl and a cyclohexyl moiety, are also showing a diminished H_3R agonistic activity, in fact they behave as partial agonists at concentrations up to 1 μM, but they turn into apparent (partial) inverse agonists at higher concentrations (10 and 100 μM). This "biphasic" behaviour is probably due to non-H_3R-mediated effects at high concentrations that interfere with an accurate estimation of the intrinsic H_3R activity of the aforementioned compounds in our cell based assay. Further experiments showed indeed that responses to **1h** and **1i** in SK-N-MC cells transfected with the human H_3R could not be antagonized by the neutral H_3R antagonist **2h** (data not shown).

The same trend in H_3R affinity shown for the alicyclic substituted ligands is followed in case of alkylation with an aromatic residue. When a benzyl group is introduced on the amino moiety of imbutamine the affinity again decreases almost 1 log unit (**1k**, Table 1), whilst the same substitution on impentamine causes only a slight decrease in the H_3R affinity (**2k**, Table 2). Benzylation of imhexamine (**3a**) to give analogue **3k** does not substantially change the H_3R affinity (Table 3). Substitution at the *para* position of phenyl rings has proven to be a successful strategy to improve affinity and/or potency of the compounds,[58] as seen with the very potent H_3R antagonist clobenpropit (Figure 1).[1,59] The H_3R affinity of the benzylated ligands **1k** and **2k** could in fact be increased via the introduction of a chlorine atom in the *para* position of the phenyl ring, as shown by ligands **1l** and **2l** (Tables 1 and 2). The latter compound is about 4-fold more active than the parent compound **2a** (Table 2), suggesting that the electron density on the phenyl ring is important for the interaction with the receptor.

Very different effects on the intrinsic H_3R activity following alkylation with (un)substituted aromatic groups can be observed as well. Benzylation of imbutamine to give compound **1k** causes a loss of intrinsic H_3R activity (α=0.72, Table 1). Further elongation of the side chain to give **2k** and **3k** produces an inversion in the intrinsic activity, thus generating partial inverse H_3R agonists (α=-0.56 and α=-0.63, Tables 2 and 3). *Para*-substitution of the benzyl ring gives

compounds **1l**, again showing "biphasic" behaviour (partial agonism up to 1μM and non-H_3R-mediated at higher concentrations), and **2l**, in which the introduction of the chlorine atom results in an increase in the potency of the compound (pEC_{50}=8.9) as well as a further decrease in the intrinsic activity, generating the only full inverse agonist (α=-0.97) identified among the ligands investigated in this study.

Incorporation of the *N*''-amino group of histamine into a pyrrolidine nucleus led to a compound with reduced potency and a maximal response that was only 56% in respect to histamine, implying that this compound is a partial H_3R agonist.[60] Similarly, incorporation of the amino group of imbutamine and impentamine in a pyrrolidine (**1o**, **2o**), piperidine (**1p**, **2p**) or azepane (**1q**, **2q**) ring generates ligands with reduced potency and intrinsic H_3R activity (Tables 1 and 2), all the compounds being partial agonists with α values ranging from 0.22 (**2q**) to 0.64 (**1p**). Ligand **2p** (Table 2), a partial agonist in this study, displaying an α value of 0.46, was previously found to behave as a full agonist (α=1.00).[41] Parallel to a diminished H_3R potency and intrinsic activity these ligands also show H_3R affinities that are decreased by 4- to 10-fold, excluding compound **2p**, for which the decrease in affinity was less marked (pK_i of 8.1 *vs* a pK_i value of 8.3 for **2a**). The only apparent relation between the structures of the compounds and their activities is that affinity and potency values are slightly higher for the impentamine derivatives **2o-q** (Table 2) than for ligands **1o-q** (Table 1), whilst an opposite behavior could be observed for the intrinsic activity, compounds **1o-q** displaying higher partial agonistic activity over **2o-q**.

When compared to our previous study,[41] three of the six compounds analyzed in that research showed a different intrinsic H_3R activity. More precisely, impentamine (**2a**) and N''-pyrrolidine-impentamine (**2p**) behaved in the present study as partial agonists (α values respectively 0.78 and 0.46, Table 2) whereas they were identified as full agonists (α=0.9 and 1.00) in the previous one. N''-i-propylimpentamine (**2d**), earlier recognized as a neutral antagonist, now behaved as a partial inverse agonist (α=-0.55, Table 2). The other three (**2i,l,m**, Table 2) showed instead values comparable to those formerly measured.

However, it is important to point out that impentamine was initially recognized as an H_3R antagonist[44] and subsequently proved to act also as a partial agonist, depending on the test system.[61]

More recently, Fox and coworkers[62] have reported the partial agonistic behavior of GT-2331 (cipralisant, Perceptin®, Figure 1), so far presumed to be an H_3R antagonist.[63] Furthermore, Gbahou et al.[64] identified proxifan (Figure 1) as a protean H_3 receptor ligand with the ability to display agonism, neutral antagonism and inverse agonism, depending on the test system. Thus, the complex pharmacodynamic profile of the discussed compounds may be caused not only by inter-assay and species differences. Additionally, the distribution of distinct receptor active states or different coupling routes in different cells or tissue may also have to be taken into account.

Recently, another histamine receptor subtype, the H_4R, was cloned by several groups and identified as a G-protein coupled receptor.[4,50,65-69] The H_4R has significant sequence homology (about 40%) to the H_3R cDNA,[68] and it shares about 60% sequence identity with the H_3R in the transmembrane regions,[50] suggesting that the two receptors might have a similar mode of ligand recognition. Although the development of selective H_3R ligands is not the primary goal of this study, the synthesized compounds were additionally screened for their affinity on the human H_4R in order to investigate H_3/H_4 receptor selectivity of the synthesised compounds. The affinity of most of the compounds is lower for the human H_4R than for the human H_3R (Tables 1-3). Impentamine (**2a**) and its ethyl (**2b**), *n*-butyl (**2e**) and *i*-butyl (**2f**) derivatives show a high selectivity for the H_3R over H_4R, the difference in affinity between the two receptors ranging between 1.6 and 1.9 log units (Table 2), but the best H_3R selectivity among the synthesised compounds is displayed by N^{α}-N^{α}-dimethylimbutamine **1m** (Table 1), with 2.4 log units difference between the two receptor's affinities (pK_i=8.5 on H_3R versus 6.1 on H_4R). Excluding ligands **1a,d,g,h,j,k,l** (Table 1) and **2g** (Table 2), which have almost the same affinity for both receptors and therefore lack H_3/H_4 receptor selectivity, all the other compounds of the series bear a selectivity that ranges between 0.5 and 1.5 log units for the H_3 over the H_4 receptor (Tables 1-3).

Conclusions

The synthesis of a series of imbutamine, impentamine and imhexamine analogues in which the primary amino function has been mono- or di-substituted with several aliphatic, alicyclic and aromatic moieties, as well as incorporated in cyclic systems, has been reported in this study. The novel ligands were pharmacologically investigated *in vitro* for their affinities on the human H_3R and H_4R subtypes, and for their intrinsic H_3R activities.

Modification of the primary amine function of **1a**, **2a** and **3a** dramatically affects the H_3R binding and functional properties of the compounds. Subtle changes in the substitution of the side chain nitrogen have great influence on the H_3R pharmacological activities of the ligands: agonists, antagonists and inverse agonists are in fact present in this series of compounds. Previously we had indeed proposed that the side chain amine function of impentamine probably interacts with the aspartate residue Asp^{114} in transmembrane domain 3,[53] and we therefore hypothesized that changes on this residue could in turn influence its agonistic properties.[41]

However, substitution with the same moiety on the three parent compounds (**1a**, **2a** and **3a**), yields ligands with different structure-activity relationships (SAR) at the H_3R, depending on the length of the side chain. This is possibly due to the existence of diverse binding pockets and a different set of receptor site points available for interaction with derivatives of **1a**, **2a** and **3a**,

that are very much dependent on the distance between the basic nitrogen of the side chain and the imidazole nucleus, as well as on the kind of substituting moiety present on the aforementioned basic nitrogen. Our group already proposed that the differences in SAR observed for the lipophilic tails of different classes of H_3R antagonists could be explained by the existence of two distinct lipophilic pockets available for antagonist binding.[53] The obtained qualitative model for the H_3R revealed a molecular determinant explaining intrinsic activity, as the conformation of the aspartic acid residue differs according to whether it is binding to agonists or antagonists.[53]

Several neutral H_3R antagonists (**1d,g,j,n**, **2g,h,n** and **3d**) have been identified within this series, but also a very potent and selective H_3R agonist (**1m**) and a very potent, though not highly selective, H_3R inverse agonist (**2l**). It has proven very difficult to draw SAR among these series of ligands, since there does not seem to be a clear correlation between the modifications applied to the side chain nitrogen and the different H_3R affinities and functional activities. Very small modifications can lead to dramatic changes in the affinity as well as in the functional activity of the generated ligands, and sometimes even to inversion of intrinsic activity, switching compounds from partial H_3R agonists to partial inverse H_3R agonists (e.g. **1k**, **2k** and **3k**). In this perspective, the side chain nitrogen can be seen as a very subtle chemical switch for the modulation of the functional activity of higher homologues of histamine on the human H_3R.

The target compounds were additionally investigated for their affinities on the human H_4R subtype in order to check selectivity, which led to the identification of N^{α}-N^{α}-dimethylimbutamine (**1m**), showing an H_3R/H_4R affinity ratio of 2.4 log units, as a potent and selective agonist for the human H_3R.

The H_3R is one of the few GPCRs that has been shown to modulate important physiological processes by means of its constitutive activity.[40] This underlines the impact of assay setup and species differencies when evaluating the behavior of ligands on the histamine H_3R, but also the usefulness of the compounds presented in this study as pharmacological tools. In view of the many potential therapeutic applications of H_3R antagonists,[16] the availability of high affinity neutral H_3R antagonists such as N^{α}-ethylimpentamine **2b** and N^{α}-cyclopentylimpentamine **2h** as well as of the potent inverse H_3R agonist N^{α}-*p*-chlorobenzylimpentamine **2l** may help in establishing whether inverse H_3R agonists or neutral H_3R antagonists will be favored for clinical applications.

Experimental procedures

General procedures. Reagents were obtained from commercial suppliers and used without further purification. Solvents used were either AR or HPLC grade. Dry THF and dichloromethane were freshly distilled from $LiAlH_4$ and CaH_2, respectively.

Compounds **1a**, **2a** and **3a** were obtained from our own laboratory stock.[44]

The 2-(ω-iodo-alkyloxy)-tetrahydro-pyrans were prepared by refluxing the corresponding 2-(ω-chloro-alkyloxy)-tetrahydro-pyrans[70] with 1.1 equivalents (eq) of sodium iodide in acetone and purified by distillation.[71,72]

Melting points were measured on an Electrothermal IA 9200 apparatus.

^{1}H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. Chemical shifts are given in ppm using the residual undeuterated solvent as reference.

Flash chromatography was performed on J.T.Baker Kieselgel 60.

All reactions were performed under an atmosphere of dry nitrogen.

5(4)-(4-Hydroxy-butyl)-imidazole-1-sulfonic acid dimethylamide (5)

2-(*tert*-Butyl-dimethyl-silanyl)-imidazole-1-sulfonic acid dimethylamide **4**[45] (8.59 g, 0.030 mol) was dissolved in THF (100 mL) and cooled to −70 °C. *n*-Butyllithium in hexane (20.35 mL, 0.033 mol) was added dropwise at −65 °C. After 30 min, a solution of 2-(4-iodo-butyloxy)-tetrahydro-pyran (8.42 g, 0.030 mol) in THF was added dropwise and the mixture was gradually allowed to warm to room temperature (r.t.) overnight. The solution was acidified with HCl (6 M) and stirred for 3 h, then the solvents were removed *in vacuo*. The residue was diluted with water, basified with K_2CO_3, extracted with dichloromethane. The organic layers were combined, washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The product was purified by flash chromatography switching gradually the eluent from ethyl acetate into ethyl acetate/methanol (9/1) to afford 2.94 g (40%) of product as a mixture of 4- and 5-isomer (10:90). ^{1}H NMR ($CDCl_3$): δ 1.70 (m, 4H, central C*H*$_2$'s 4- + 5-isomer), 1.93 (s, 1H, O*H*, 4- + 5-isomer), 2.60 + 2.75 (t, 2H, imidazole-5(4)-C*H*$_2$, 4- + 5-isomer), 2.80 + 2.85 (s, 6H, N(C*H*$_3$)$_2$, 4- + 5-isomer), 3.65 (t, 2H, C*H*$_2$OH 4- + 5-isomer), 6.80 + 6.92 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.80 + 7.85 (s, 1H, imidazole-2H, 4- + 5-isomer).

5(4)-(5-Hydroxy-pentyl)-imidazole-1-sulfonic acid dimethylamide (6)

Using the same procedure as for **5** with 9.73 g (0.034 mol) of **4** and 10.00 g (0.034 mol) of 2-(5-iodo-pentyloxy)-tetrahydro-pyran gave 3.07 g (35%) of product as a mixture of 4- and 5-isomer (14:86). ^{1}H NMR ($CDCl_3$): δ 1.55 (m, 6H, central C*H*$_2$'s 4- + 5-isomer), 2.50 + 2.68 (t, 2H, imidazole-5(4)-C*H*$_2$, 4- + 5-isomer), 2.78 + 2.82 (s, 6H, N(C*H*$_3$)$_2$, 4- + 5-isomer), 3.58 (t, 2H, C*H*$_2$OH 4- + 5-isomer), 6.78 + 6.89 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.75 + 7.78 (s, 1H, imidazole-2H, 4- + 5-isomer).

5(4)-(4-Oxo-butyl)-imidazole-1-sulfonic acid dimethylamide (7)

A solution of oxalyl chloride (2.27 g, 0.018 mol) in anhydrous dichloromethane (50 mL) was cooled to –60 °C. A solution of DMSO (2.79 g, 0.036 mol) in anhydrous dichloromethane (10 mL) was added dropwise. After stirring for 10 min, a solution of **5** (2.94 g, 0.012 mol) in dichloromethane (15 mL) was added dropwise. After additional stirring for 15 min, triethylamine (9.03 g, 0.089 mol) was added. The mixture was allowed to warm to r.t. and poured into water. The aqueous layer was extracted with dichloromethane and the combined organic layers were washed successively with dilute sodium carbonate (5%), water and brine, then dried (($MgSO_4$) and concentrated in vacuo to yield 2.72 g (93%) of product as a mixture of 4- and 5-isomer (12.5:87.5). ^{1}H NMR ($CDCl_3$): δ 1.95 (m, 4H, central C*H*$_2$'s, 4- + 5-isomer), 2.50 (m, 2H, C*H*$_2$CHO 4- + 5-isomer), 2.70 (t, 2H, imidazole-5(4)-C*H*$_2$, 4- + 5-isomer), 2.78 + 2.82 (s, 6H, N(C*H*$_3$)$_2$, 4- + 5-isomer), 6.78 + 6.90 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.75 + 7.80 (s, 1H, imidazole-2H, 4- + 5-isomer), 9.70 + 9.75 (t, 1H, C*H*O 4- + 5-isomer).

5(4)-(5-Oxo-pentyl)-imidazole-1-sulfonic acid dimethylamide (8)

Using the same procedure as for **7** with 3.22 g (0.012 mol) of **6** yielded 2.88 g (90%) of product as a mixture of 4- and 5-isomer (28.5:71.5). ^{1}H NMR ($CDCl_3$): δ 1.70 (m, 4H, central C*H*$_2$'s, 4- + 5-isomer), 2.48 (m, 2H, C*H*$_2$CHO 4- + 5-isomer), 2.70 (t, 2H, imidazole-5(4)-C*H*$_2$, 4- + 5-isomer), 2.80 + 2.82 (s, 6H, N(C*H*$_3$)$_2$, 4- + 5-isomer), 6.80 + 6.92 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.78 + 7.80 (s, 1H, imidazole-2H, 4- + 5-isomer), 9.78 (t, 1H, C*H*O 4- + 5-isomer).

5-Hydroxy-pentanoic acid methyl ester (16)

δ-valerolactone (47.17 g, 0.47 mol) in methanol (300 mL) and concentrated H_2SO_4 (4 mL) was heated to reflux for 16 h. The mixture was cooled to r.t. and the solvent was removed *in vacuo*. The residue was dissolved in dichloromethane, washed three times with a saturated solution of $NaHCO_3$, dried over $MgSO_4$ and evaporated to dryness yielding 46.91 g (76%) of product. ^{1}H NMR ($CDCl_3$): δ 1.48-1.80 (m, 4H, central C*H*$_2$'s), 2.30 (m, 2H, C*H*$_2$COOCH$_3$), 3.58 (m, 3H, C*H*$_2$OH + C*H*$_3$OCO).

6-Hydroxy-hexanoic acid methyl ester (17)

Using the same procedure as for **16** with 50.1 g (0.44 mol) of ε-caprolactone yielded 39.38 g (62%) of product. ^{1}H NMR ($CDCl_3$): δ 1.28-1.70 (m, 4H, central C*H*$_2$'s), 2.28 (m, 2H, C*H*$_2$COOCH$_3$), 3.50-3.68 (m, 3H, C*H*$_2$OH + C*H*$_3$OCO).

5-Oxo-pentanoic acid methyl ester (18)

Using the same procedure as for **7** with 46.91 g (0.36 mol) of **16** yielded 33.15 g (72%) of product. ^{1}H NMR ($CDCl_3$): δ 1.80-2.00 (m, 2H, central CH_2), 2.35 (m, 2H, CH_2CHO), 2.50 (m, 2H, CH_2COOCH_3), 3.63 (s, 3H, CH_3OCO), 9.75 (t, 1H, CHO).

6-Oxo-hexanoic acid methyl ester (19)

Using the same procedure as for **7** with 39.38 g (0.27 mol) of **17** yielded 31.68 g (82%) of product. ^{1}H NMR ($CDCl_3$): δ 1.52-1.75 (m, 4H, central CH_2's), 2.30 (m, 2H, CH_2CHO), 2.43 (m, 2H, CH_2COOCH_3), 3.65 (s, 3H, CH_3OCO), 9.78 (t, 1H, CHO).

4-[4-(Toluene-4-sulfonyl)-4,5-dihydro-oxazol-5-yl]-butyric acid methyl ester (20)

To a stirred suspension of tosyl methyl isocyanide (TosMIC) (49.68 g, 0.25 mol) and **18** (33.15 g, 0.25 mol) in absolute ethanol (800 mL) was added finely powdered sodium cyanide (1.24 g, 0.025 mol). The yellow-orange suspension was stirred for 30 min, filtered and washed with hexane/ether (1:1), to give 40.06 g (49%) of product. ^{1}H NMR ($CDCl_3$): δ 1.75 (m, 4H, 2 × CH_2), 2.36 (m, 2H, 1 × CH_2), 2.46 (s, 3H, *p*-CH_3), 3.55 (s, 3H, CH_3OCO), 4.79 (d, 1H, oxazoline-4H), 5.05 (q, 1H, oxazoline-5H), 6.95 (s, 1H, oxazoline-2H), 7.35 (d, 2H, 2,6-phenyl-H), 7.80 (d, 2H, 3,5-phenyl-H).

5-[4-(Toluene-4-sulfonyl)-4,5-dihydro-oxazol-5-yl]-pentanoic acid methyl ester (21)

Using the same procedure as for **20** with 31.68 g (0.22 mol) of **19** 54.17 g (73%) of product was obtained. ^{1}H NMR ($CDCl_3$): δ 1.30-1.75 (m, 6H, 3 × CH_2), 2.29 (m, 2H, 1 × CH_2), 2.43 (s, 3H, *p*-CH_3), 3.53 (s, 3H, CH_3OCO), 4.73 (d, 1H, oxazoline-4H), 5.03 (q, 1H, oxazoline-5H), 6.95 (s, 1H, oxazoline-2H), 7.38 (d, 2H, 2,6-phenyl-H), 7.78 (d, 2H, 3,5-phenyl-H).

4-(1*H*-Imidazol-4-yl)-butyric acid ethyl ester (22)

In a re-sealable pressure tube, a solution of **20** in absolute ethanol saturated with ammonia was heated between 120-140 °C. After 20 h the reaction mixture was cooled to r.t. and the solvent was removed *in vacuo*. The residue (45.67 g) was dissolved in HCl 37% (300 mL) and heated to reflux for 16 h. The mixture was cooled to r.t., evaporated to dryness, dissolved in water and washed with ether, then concentrated *in vacuo*. The residue in ethanol (300 mL) and concentrated H_2SO_4 (ca. 3 mL) was heated to reflux. After 16 h the mixture was cooled to r.t. and evaporated to dryness. The residue was dissolved in water and washed with ether, then the water layer was basified with $NaHCO_3$ and extracted with dichloromethane. The combined organic extracts were dried over $MgSO_4$ and evaporated *in vacuo* yielding 18.46 g (82%) of product. ^{1}H NMR ($CDCl_3$): δ 1.20 (t, 3H, CH_3CH_2OCO), 1.95 (m, 2H, central CH_2),

2.32 (t, 2H, CH_2COO), 2.62 (t, 2H, imidazole-CH_2), 4.05 (q, 2H, CH_3CH_2OCO), 6.78 (s, 1H, imidazole-5H), 7.65 (s, 1H, imidazole-2H), 10.08 (s, 1H, imidazole-N*H*).

5-(1*H*-Imidazol-4-yl)-pentanoic acid ethyl ester (23)

Using the same procedure as for **22** with 54.17 g (0.16 mol) of **21** gave 15.90 g (51%) of product. ^{1}H NMR (DMSO): δ 1.15 (t, 3H, CH_3CH_2OCO), 1.50 (m, 4H, central CH_2's), 2.29 (t, 2H, CH_2COO), 2.45 (t, 2H, imidazole-CH_2), 4.00 (q, 2H, CH_3CH_2OCO), 6.65 (s, 1H, imidazole-5H), 7.45 (s, 1H, imidazole-2H), 11.70 (s, 1H, imidazole-N*H*).

4-(1-Trityl-1*H*-Imidazol-4-yl)-butyric acid ethyl ester (24)

22 (15.20 g, 0.083 mol) was dissolved in dichloromethane (200 mL). Triphenylmethyl chloride (23.03 g, 0.083 mol) in dichloromethane (200 mL) was added dropwise, followed by triethylamine (17.18 mL, 0.12 mol). The reaction mixture was stirred at r.t. overnight, then it was washed successively with K_2CO_3 1M and water. The organic layer was dried over $MgSO_4$ and then concentrated under reduced pressure to give 34.22 g (97%) of product. ^{1}H NMR ($CDCl_3$): δ 1.20 (t, 3H, CH_3CH_2OCO), 1.95 (m, 2H, central CH_2), 2.30 (t, 2H, CH_2COO), 2.55 (t, 2H, imidazole-CH_2), 4.05 (q, 2H, CH_3CH_2OCO), 6.50 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

5-(1-Trityl-1*H*-Imidazol-4-yl)-pentanoic acid ethyl ester (25)

Using the same procedure as for **24** with 15.90 g (0.081 mol) of **23** gave 33.75 g (95%) of product. ^{1}H NMR ($CDCl_3$): δ 1.20 (t, 3H, CH_3CH_2OCO), 1.62 (m, 4H, central CH_2's), 2.28 (t, 2H, CH_2COO), 2.50 (t, 2H, imidazole-CH_2), 4.05 (q, 2H, CH_3CH_2OCO), 6.50 (s, 1H, imidazole-5H), 7.00-7.35 (m, 16H, imidazole-2H + Ph_3C).

4-(1-Trityl-1*H*-Imidazol-4-yl)-butan-1-ol (26)

A suspension of **24** (34.22 g, 0.081 mol) in THF (200 mL) was added, with efficient stirring, to an ice-cold suspension of $LiAlH_4$ (3.06 g, 0.081 mol) in THF (50 mL). The reaction mixture was stirred for 15 min at r.t.. Next, water (5 mL), followed by saturated K_2CO_3solution (5 mL) were added dropwise, filtered and concentrated *in vacuo* to afford 25.81 g (84%) of product. ^{1}H NMR ($CDCl_3$): δ 1.50-1.78 (m, 4H, central CH_2's), 2.50 (t, 2H, imidazole-CH_2), 3.58 (t, 2H, CH_2OH), 6.50 (s, 1H, imidazole-5H), 7.00-7.40 (m, 16H, imidazole-2H + Ph_3C).

5-(1-Trityl-1*H*-Imidazol-4-yl)-pentan-1-ol (27)

Using the same procedure as for **26** with 33.75 g (0.077 mol) of **25** gave 25.45 g (83%) of product. ^{1}H NMR ($CDCl_3$): δ 1.30-1.70 (m, 6H, central CH_2's), 2.50 (t, 2H, imidazole-CH_2), 3.58 (t, 2H, CH_2OH), 6.45 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

4-(1-Trityl-1*H*-Imidazol-4-yl)-butyraldehyde (28)

Using the same procedure as for **7** with 25.81 g (0.067 mol) of **26** yielded 12.61 g (49%) of product. ^{1}H NMR ($CDCl_3$): δ 1.95 (m, 2H, central CH_2), 2.45 (m, 2H, CH_2CHO), 2.55 (t, 2H, imidazole-CH_2), 6.52 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C), 9.70 (t, 1H, *CH*O).

5-(1-Trityl-1*H*-Imidazol-4-yl)-pentanal (29)

Using the same procedure as for **7** with 25.45 g (0.064 mol) of **27** yielded 17.68 g (70%) of product. ^{1}H NMR ($CDCl_3$): δ 1.55-1.75 (m, 4H, central CH_2's), 2.42 (m, 2H, CH_2CHO), 2.55 (t, 2H, imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 7.03-7.35 (m, 16H, imidazole-2H + Ph_3C), 9.70 (t, 1H, *CH*O).

General procedures for the synthesis of the protected aminoalkylimidazoles 13c,k,n, 14c,d,i,k,n, 15d,i,k, 30b,d-j,l,m,o-q and 31b,e-h,j,l,m,o-q

Method A. 1 eq of **9**, **10**[73] or **11**[44] was mixed with 1.1 eq of benzaldehyde (**1k**, **2k** and **3k**), 2.5 eq of cyclohexanone (**3i**) or 2.5 eq of acetone (**3d**) and a catalytic amount of PtO_2 in absolute ethanol, sealed in a stainless steel bomb and stirred under a hydrogen atmosphere (50 bar) at r.t.. After 3 days the reaction mixture was filtered through a pad of Celite® and the filtrate was concentrated *in vacuo*. The residue was purified by flash chromatography by washing with ethyl acetate/methanol (9/1) as eluent. The product was subsequently eluted switching gradually the eluent from methanol into methanol/ammonia (95/5).

Method B. 1 eq of **12**[73] was mixed with 3 eq of the appropriate amine and heated at 100 °C. After 48 h the excess amine was evaporated under reduced pressure and the residue purified by flash chromatography as described in Method A.

Method C (reductive amination). 1 eq of the required aldehyde (**7**, **8, 28** or **29**) was stirred for 10 min with the appropriate amine (3 eq in case of primary amines, 1 eq in case of secondary amines) and 1 eq of acetic acid in dichloroethane at r.t., then 1.5 eq of sodium triacetoxyborohydryde was added and stirred overnight. Water was added and stirred for 15 min. Dichloroethane was removed *in vacuo*, the residue basified with K_2CO_3 and extracted with chloroform. The combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was then purified by flash chromatography as described in Method A.

5(4)-(4-Propylamino-butyl)-imidazole-1-sulfonic acid dimethylamide (13c)

Method C with aldehyde **7** and *n*-propylamine; yield 0.49 g (30%). Mixture of 4- and 5-isomer (9:91). ^{1}H NMR ($CDCl_3$): δ 0.82 (t, 3H, CH_3 4- + 5-isomer), 1.30-1.72 (m, 6H, $CH_2CH_2CH_3$ 4- and 5-isomer + central CH_2's, 4- and 5-isomer), 2.40-2.60 (m, 4H, CH_2NHCH_2 4- + 5-isomer), 2.68 (t, 2H, imidazole-5(4)-CH_2, 4- + 5-isomer), 2.78 + 2.82 (s, 6H, $N(CH_3)_2$, 4- + 5-isomer),

6.78 + 6.88 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.72 + 7.78 (s, 1H, imidazole-2H, 4- + 5-isomer).

5(4)-(4-Benzylamino-butyl)-imidazole-1-sulfonic acid dimethylamide (13k)

Method A with compound **9** and benzaldehyde; yield 1.53 g (33%). ^{1}H NMR ($CDCl_3$): δ 1.20-1.90 (m, 4H, central CH_2's), 2.55 (m, 4H, CH_2NH + imidazole-5(4)-CH_2), 2.78 (s, 6H, $N(CH_3)_2$), 3.90 (s, 2H, CH_2-phenyl), 6.78 (s, 1H, imidazole-4(5)H), 7.20-7.50 (m, 5H, phenyl-H), 7.80 (s, 1H, imidazole-2H).

5(4)-(4-Diethylamino-butyl)-imidazole-1-sulfonic acid dimethylamide (13n)

Method C with aldehyde **7** and diethylamine; yield 0.56 g (33%). Mixture of 4- and 5-isomer (11:89). ^{1}H NMR ($CDCl_3$): δ 0.98 (t, 6H, $(CH_3CH_2)_2$ 4- + 5-isomer), 1.30-1.70 (m, 4H, central CH_2's, 4- + 5-isomer), 2.30-2.55 (m, 6H, $CH_2N(CH_2)_2$ 4- + 5-isomer), 2.70 (t, 2H, imidazole-5(4)-CH_2, 4- + 5-isomer), 2.78 + 2.82 (s, 6H, $N(CH_3)_2$, 4- + 5-isomer), 6.78 + 6.90 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.78 + 7.80 (s, 1H, imidazole-2H, 4- + 5-isomer).

5(4)-(5-Propylamino-pentyl)-imidazole-1-sulfonic acid dimethylamide (14c)

Method C with aldehyde **8** and *n*-propylamine; yield 0.34 g (20%). Mixture of 4- and 5-isomer (20:80). ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H, CH_3 4- + 5-isomer), 1.30-1.78 (m, 8H, $CH_2CH_2CH_3$ 4- and 5-isomer + central CH_2's, 4- and 5-isomer), 2.45-2.62 (m, 4H, CH_2NHCH_2 4- + 5-isomer), 2.68 (t, 2H, imidazole-5(4)-CH_2, 4- + 5-isomer), 2.78 + 2.82 (s, 6H, $N(CH_3)_2$, 4- + 5-isomer), 6.78 + 6.90 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.75 + 7.80 (s, 1H, imidazole-2H, 4- + 5-isomer).

5(4)-(5-Cyclohexylamino-pentyl)-imidazole-1-sulfonic acid dimethylamide (14i)

Method B with compound **12** and cyclohexylamine; yield 2.0 g (41%). ^{1}H NMR ($CDCl_3$): δ 1.06-2.23 (m, 17H), 2.69 (t, 2H, imidazole-5(4)-CH_2), 2.79-2.93 (m, 8H, + CH_2NH + $N(CH_3)_2$), 6.79 (s, 1H, imidazole-4(5)H), 7.83 (s, 1H, imidazole-2H).

5(4)-(5-Benzylamino-pentyl)-imidazole-1-sulfonic acid dimethylamide (14k)

Method A with compound **10** and benzaldehyde; yield 1.14 g (57%). ^{1}H NMR ($CDCl_3$): δ 1.20-1.90 (m, 6H, central CH_2's), 2.55-2.70 (m, 4H, CH_2NH + imidazole-5(4)-CH_2), 2.82 (s, 6H, $N(CH_3)_2$), 3.90 (s, 2H, CH_2-phenyl), 6.80 (s, 1H, imidazole-4(5)H), 7.20-7.50 (m, 5H, phenyl-H), 7.75 (s, 1H, imidazole-2H).

5(4)-(5-Diethylamino-pentyl)-imidazole-1-sulfonic acid dimethylamide (14n)

Method C with aldehyde **8** and diethylamine; yield 0.87 g (48%). Mixture of 4- and 5-isomer (20:80). ^{1}H NMR ($CDCl_3$): δ 0.98 (t, 6H, $(CH_3CH_2)_2$ 4- + 5-isomer), 1.25-1.72 (m, 6H, central CH_2's, 4- + 5-isomer), 2.30-2.58 (m, 6H, $CH_2N(CH_2)_2$ 4- + 5-isomer), 2.68 (t, 2H, imidazole-5(4)-CH_2, 4- + 5-isomer), 2.80 + 2.85 (s, 6H, $N(CH_3)_2$, 4- + 5-isomer), 6.80 + 6.90 (s, 1H, imidazole-4(5)H, 4- + 5-isomer), 7.78 + 7.80 (s, 1H, imidazole-2H, 4- + 5-isomer).

5(4)-(6-Isopropylamino-hexyl)-imidazole-1-sulfonic acid dimethylamide (15d)

Method A with compound **11** and acetone; yield 3.75 g (65%). ^{1}H NMR ($CDCl_3$): δ 1.00 (d, 6H, $(CH_3)_2CH$), 1.38-1.72 (m, 8H, central CH_2's), 2.30 (s, 1H, NH), 2.53 (m, 4H, imidazole-5(4)-CH_2 + CH_2NH), 2.70 (m, 1H, $CH(CH_3)_2$), 2.78 (s, 6H, $N(CH_3)_2$), 6.78 (s, 1H, imidazole-4(5)H), 7.80 (s, 1H, imidazole-2H).

5(4)-(6-Cyclohexylamino-hexyl)-imidazole-1-sulfonic acid dimethylamide (15i)

Method A with compound **11** and cyclohexanone; yield 4.2 g (52%). ^{1}H NMR ($CDCl_3$): δ 0.80-1.30 (m, 10H), 1.30-1.90 (m, 8H), 2.30 (m, 1H, NHCH), 2.50 (m, 4H, imidazole-5(4)-CH_2 + CH_2NH), 2.78 (s, 6H, $N(CH_3)_2$), 6.78 (s, 1H, imidazole-4(5)H), 7.80 (s, 1H, imidazole-2H).

5(4)-(6-Benzylamino-hexyl)-imidazole-1-sulfonic acid dimethylamide (15k)

Method A with compound **11** and benzaldehyde; yield 1.21 g (49%). ^{1}H NMR ($CDCl_3$): δ 1.20-1.70 (m, 8H, central CH_2's), 2.50-2.70 (m, 4H, CH_2NH + imidazole-5(4)-CH_2), 2.90 (s, 6H, $N(CH_3)_2$), 3.90 (s, 2H, CH_2-phenyl), 6.80 (s, 1H, imidazole-4(5)H), 7.20-7.50 (m, 5H, phenyl-H), 7.75 (s, 1H, imidazole-2H).

Ethyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30b)

Method C with aldehyde **28** and ethylamine; yield 0.064 g (6%). ^{1}H NMR ($CDCl_3$): δ 1.05 (t, 3H, CH_3CH_2), 1.38-1.70 (m, 4H, central CH_2's), 2.38-2.68 (m, 6H, CH_2NHCH_2 + imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 6.98-7.38 (m, 16H, imidazole-2H + Ph_3C).

Isopropyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30d)

Method C with aldehyde **28** and isopropylamine; yield 0.51 g (45%). ^{1}H NMR ($CDCl_3$): δ 1.00 (d, 6H, $(CH_3)_2CH$), 1.38-1.72 (m, 4H, central CH_2's), 2.30 (s, 1H, NH), 2.53 (m, 4H, imidazole-CH_2 + CH_2NH), 2.70 (m, 1H, $CH(CH_3)_2$), 6.45 (s, 1H, imidazole-5H), 6.95-7.32 (m, 16H, imidazole-2H + Ph_3C).

Butyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30e)

Method C with aldehyde **28** and *n*-butylamine; yield 0.40 g (34%). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 3H, CH_3), 1.20-1.80 (m, 8H, 4 × CH_2), 2.55 (m, 6H, CH_2NHCH_2 + imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 7.05-7.40 (m, 16H, imidazole-2H + Ph_3C).

Isobutyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30f)

Method C with aldehyde **28** and isobutylamine; yield 1.49 g (59%). ^{1}H NMR ($CDCl_3$): δ 0.85 (d, 6H, $(CH_3)_2CH$), 1.40-1.82 (m, 5H, $CH(CH_3)_2$ + central CH_2's), 2.30 (s, 1H, NH), 2.38 (d, 2H, $CHCH_2NH$), 2.55 (m, 4H, imidazole-CH_2 + CH_2NH), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

***tert*-Butyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30g)**

Method C with aldehyde **28** and *tert*-butylamine; yield 0.23 g (20%). ^{1}H NMR ($CDCl_3$): δ 1.05 (s, 9H, $(CH_3)_3C$), 1.40-1.75 (m, 4H, central CH_2's), 2.55 (m, 4H, imidazole-CH_2 + CH_2NH), 6.50 (s, 1H, imidazole-5H), 7.00-7.35 (m, 16H, imidazole-2H + Ph_3C).

Cyclopentyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30h)

Method C with aldehyde **28** and cyclopentylamine; yield 0.15 g (13%). ^{1}H NMR ($CDCl_3$): δ 1.20-1.90 (m, 12H), 2.55 (m, 5H, imidazole-CH_2 + CH_2NH + NHCH), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

Cyclohexyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30i)

Method C with aldehyde **28** and cyclohexylamine; yield 0.34 g (27%). ^{1}H NMR ($CDCl_3$): δ 0.80-1.30 (m, 6H), 1.30-1.90 (m, 8H), 2.00 (s, 1H, NH), 2.30 (m, 1H, NHCH), 2.50 (m, 4H, imidazole-CH_2 + CH_2NH), 6.45 (s, 1H, imidazole-5H), 6.90-7.32 (m, 16H, imidazole-2H + Ph_3C).

Cycloheptyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30j)

Method C with aldehyde **28** and cycloheptylamine; yield 0.15 g (12%). ^{1}H NMR ($CDCl_3$): δ 1.28-1.90 (m, 16H), 2.45-2.70 (m, 5H), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

4-Chloro-benzyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30l)

Method C with aldehyde **28** and 4-chlorobenzylamine; yield 0.13 g (10%). ^{1}H NMR ($CDCl_3$): δ 1.35-1.75 (m, 4H, central CH_2's), 2.55 (m, 4H, CH_2NH + imidazole-CH_2), 3.70 (s, 2H, CH_2-phenyl), 6.50 (s, 1H, imidazole-5H), 6.90-7.38 (m, 20H, imidazole-2H + phenyl-H + Ph_3C).

Dimethyl-[4-(1-trityl-1*H*-imidazol-4-yl)-butyl]-amine (30m)

Method C with aldehyde **28** and dimethylamine; yield 0.48 g (45%). ^{1}H NMR ($CDCl_3$): δ 1.30-1.68 (m, 4H, central CH_2's), 2.10 (s, 6H, $N(CH_3)_2$), 2.18 (t, 2H, CH_2NH), 2.48 (t, 2H, imidazole-CH_2), 6.45 (s, 1H, imidazole-5H), 6.95-7.30 (m, 16H, imidazole-2H + Ph_3C).

4-(4-Pyrrolidin-1-yl-butyl)-1-trityl-1*H*-imidazole (30o)

Method C with aldehyde **28** and pyrrolidine; yield 0.53 g (26%). ^{1}H NMR ($CDCl_3$): δ 1.40-1.80 (m, 8H), 2.30-2.60 (m, 8H), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

1-[4-(1-Trityl-1*H*-imidazol-4-yl)-butyl]-piperidine (30p)

Method C with aldehyde **28** and piperidine; yield 0.75 g (26%). ^{1}H NMR ($CDCl_3$): δ 1.30-1.90 (m, 11H), 2.28 (m, 5H), 2.53 (t, 2H, imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 7.15-7.40 (m, 16H, imidazole-2H + Ph_3C).

1-[4-(1-Trityl-1*H*-imidazol-4-yl)-butyl]-azepane (30q)

Method C with aldehyde **28** and azepane; yield 0.24 g (20%). ^{1}H NMR ($CDCl_3$): δ 1.30-1.70 (m, 12H), 2.30-2.60 (m, 8H, $CH_2N(CH_2)_2$ + imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 6.90-7.35 (m, 16H, imidazole-2H + Ph_3C).

Ethyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31b)

Method C with aldehyde **29** and ethylamine; yield 0.98 g (61%). ^{1}H NMR ($CDCl_3$): δ 1.10 (t, 3H, CH_3CH_2), 1.25-1.68 (m, 6H, central CH_2's), 2.45-2.70 (m, 6H, CH_2NHCH_2 + imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

5(4)-(5-Isopropylamino-pentyl)-imidazole-1-sulfonic acid dimethylamide (31d)

Method B with compound **12** and isopropylamine; yield 1.50 g (36%). ^{1}H NMR ($CDCl_3$): δ 1.44-1.53 (m, 10H, $(CH_3)_2CH$ + 2 × CH_2), 1.57-1.75 (m, 2H, 1 × CH_2), 1.81-2.01 (m, 2H, 1 × CH_2), 2.68 (t, 2H, imidazole-5(4)-CH_2), 2.82-2.92 (m, 9H, CH_2NH + $N(CH_3)_2$ + $CH(CH_3)_2$), 6.79 (s, 1H, imidazole-4(5)H), 7.82 (s, 1H, imidazole-2H).

Butyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31e)

Method C with aldehyde **29** and *n*-butylamine; yield 0.98 g (61%). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 3H, CH_3), 1.20-1.70 (m, 10H, 5 × CH_2), 2.52 (m, 6H, CH_2NHCH_2 + imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 7.08-7.42 (m, 16H, imidazole-2H + Ph_3C).

Isobutyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31f)

Method C with aldehyde **29** and isobutylamine; yield 0.75 g (33%). ^{1}H NMR ($CDCl_3$): δ 0.88 (d, 6H, $(CH_3)_2CH$), 1.18-1.80 (m, 7H, $CH(CH_3)_2$ + central CH_2's), 2.38 (d, 2H, $CHCH_2NH$), 2.55 (m, 4H, imidazole-CH_2 + CH_2NH), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

***tert*-Butyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31g)**

Method C with aldehyde **29** and *tert*-butylamine; yield 0.52 g (43%). ^{1}H NMR ($CDCl_3$): δ 1.08 (s, 9H, $(CH_3)_3C$), 1.25-1.70 (m, 6H, central CH_2's), 2.50 (m, 4H, imidazole-CH_2 + CH_2NH), 6.48 (s, 1H, imidazole-5H), 7.05-7.35 (m, 16H, imidazole-2H + Ph_3C).

Cyclopentyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31h)

Method C with aldehyde **29** and cyclopentylamine; yield 1.44 g (81%). ^{1}H NMR ($CDCl_3$): δ 1.20-1.90 (m, 12H), 2.55 (m, 4H), 2.88-3.18 (m, 3H), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

Cycloheptyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31j)

Method C with aldehyde **29** and cycloheptylamine; yield 0.89 g (71%). ^{1}H NMR ($CDCl_3$): δ 1.20-1.90 (m, 16H), 2.20-2.68 (m, 7H), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

4-Chloro-benzyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31l)

Method A with aldehyde **29** and 4-chlorobenzylamine; yield 0.57 g (20%). ^{1}H NMR ($CDCl_3$): δ 1.18-1.70 (m, 6H, central CH_2's), 2.05 (s, 1H, N*H*), 2.50 (m, 4H, CH_2NH + imidazole-CH_2), 3.68 (s, 2H, CH_2-phenyl), 6.47 (s, 1H, imidazole-5H), 6.95-7.38 (m, 20H, imidazole-2H + phenyl-H + Ph_3C).

Dimethyl-[5-(1-trityl-1*H*-imidazol-4-yl)-pentyl]-amine (31m)

Method C with aldehyde **29** and dimethylamine; yield 0.75 g (35%). ^{1}H NMR ($CDCl_3$): δ 1.18-1.70 (m, 6H, central CH_2's), 2.05-2.23 (m, 9H, $N(CH_3)_2$ + CH_2NH), 2.49 (t, 2H, imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 7.00-7.35 (m, 16H, imidazole-2H + Ph_3C).

4-(5-Pyrrolidin-1-yl-pentyl)-1-trityl-1*H*-imidazole (31o)

Method C with aldehyde **29** and pyrrolidine; yield 0.46 g (41 %). ^{1}H NMR ($CDCl_3$): δ 1.20-1.82 (m, 8H), 2.35-2.65 (m, 10H), 6.48 (s, 1H, imidazole-5H), 7.00-7.38 (m, 16H, imidazole-2H + Ph_3C).

1-[5-(1-Trityl-1*H*-imidazol-4-yl)-pentyl]-piperidine (31p)

Method C with aldehyde **29** and piperidine; yield 0.60 g (28%). ^{1}H NMR ($CDCl_3$): δ 1.12-1.70 (m, 12H), 2.10-2.35 (m, 6H), 2.45 (t, 2H, imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 6.95-7.35 (m, 16H, imidazole-2H + Ph_3C).

1-[5-(1-Trityl-1*H*-imidazol-4-yl)-pentyl]-azepane (31q)

Method C with aldehyde **29** and azepane; yield 1.24 g (68%). ^{1}H NMR ($CDCl_3$): δ 1.18-1.72 (m, 14H), 2.35-2.70 (m, 8H, CH_2N(CH_2)$_2$ + imidazole-CH_2), 6.48 (s, 1H, imidazole-5H), 7.15-7.40 (m, 16H, imidazole-2H + Ph_3C).

General procedures for the deprotection of the final aminoalkylimidazoles 1b-q, 2b-q and 3d,i,k

Method D (removal of the trityl group). 1 eq of the protected aminoalkylimidazole was mixed with 10 eq of hydrochloric acid (1 N) (**1b,d,e-j,l,m,o-q** and **2b,e-h,l,m,o-q**) or hydrobromic acid (1 N) (**2j**) and heated under reflux. After 2 h the mixture was cooled down, washed with diethyl ether and concentrated *in vacuo*. The salt was either washed with diethyl ether, re-crystallized from ethanol/ethyl acetate or ethanol/diethyl ether or converted into the oxalic acid or L(+)-tartaric acid salt according to the following procedure. The residue was dissolved in water, basified with K_2CO_3 and extracted with $CHCl_3$. The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. The free base was dissolved in ethyl acetate with a few drops of methanol, then added dropwise to a solution of 2 eq of oxalic acid dihydrate or L(+)-tartaric acid in ethyl acetate/methanol. The precipitate was collected by filtration.

Method E (removal of the dimethylsulfamoyl group). The same procedure as described in method A was adopted, but 30% HBr (**1c,k,n**, **2c,d,i,k,n** and **3k**) or 37% HCl (**3d,i**) was used and the mixture was refluxed for 16 h. The dihydrobromic or dihydrochloric acid salt was either washed with diethyl ether, re-crystallized from ethanol/ethyl acetate or ethanol/diethyl ether or converted into the dioxalate or ditartrate salt as described above.

Ethyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine oxalic acid salt (1b)

Yield 0.011 g (20%). White powder. Mp: 135-137 °C. ^{1}H NMR (D_2O): δ 1.20 (t, 3H, CH_3CH$_2$), 1.58-1.85 (m, 4H, central CH_2's), 2.70 (m, 2H, imidazole-CH_2), 2.90-3.18 (m, 4H, CH_2NHCH_2), 7.20 (s, 1H, imidazole-5H), 8.55 (s, imidazole-2H).

[4-(1*H*-Imidazol-4-yl)-butyl]-propyl-amine oxalic acid salt (1c)

Yield 0.46 g (77%). White powder. Mp: 156-158 °C. ^{1}H NMR (D_2O): δ 0.90 (t, 3H, $CH_3CH_2CH_2$), 1.50-1.80 (m, 6H, $CH_3CH_2CH_2$ + central CH_2's), 2.72 (t, 2H, imidazole-CH_2), 2.85-3.15 (m, 4H, CH_2NHCH_2), 7.19 (s, 1H, imidazole-5H), 8.50 (s, imidazole-2H).

[4-(1*H*-imidazol-4-yl)-butyl]-isopropyl-amine oxalic acid salt (1d)

Yield 0.18 g (41%). White powder. Mp: 165-166 °C. ^{1}H NMR (D_2O): δ 1.20 (d, 6H, $(CH_3)_2CH$), 1.68 (m, 4H, central CH_2's), 2.75 (t, 2H, imidazole-CH_2), 3.02 (m, 2H, CH_2NH), 3.35 (m, 1H, $CH(CH_3)_2$), 7.20 (s, 1H, imidazole-5H), 8.52 (s, 1H, imidazole-2H).

Butyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine oxalic acid salt (1e)

Yield 0.19 g (56%). White powder. Mp: 149-150 °C. ^{1}H NMR (D_2O): δ 0.85 (t, 3H, CH_3), 1.20-1.42 (m, 2H, 1 × CH_2), 1.48-1.78 (m, 6H, 3 × CH_2), 2.69 (t, 2H, imidazole-CH_2), 2.95 (m, 4H, CH_2NHCH_2), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

[4-(1*H*-imidazol-4-yl)-butyl]-isobutyl-amine dihydrochloride (1f)

Yield 0.57 g (62%). Light yellow powder. Mp: 158-161 °C. ^{1}H NMR (D_2O): δ 0.95 (d, 6H, $(CH_3)_2CH$), 1.70 (m, 4H, central CH_2's), 1.95 (m, 1H, $CH(CH_3)_2$), 2.75 (t, 2H, imidazole-CH_2), 2.83 (d, 2H, $CHCH_2NH$), 3.00 (m, 2H, CH_2NH), 7.20 (s, 1H, imidazole-5H), 8.52 (m, 1H, imidazole-2H).

***tert*-Butyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine oxalic acid salt (1g)**

Yield 0.13 g (64%). White powder. Mp: 138-141 °C. ^{1}H NMR (D_2O): δ 1.28 (s, 9H, $(CH_3)_3C$), 1.68 (m, 4H, central CH_2's), 2.75 (t, 2H, imidazole-CH_2), 2.98 (t, 2H, CH_2NH), 7.18 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

Cyclopentyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine tartaric acid salt (1h)

Yield 0.068 g (41%). Dark yellow powder. Mp: 70-72 °C. ^{1}H NMR (D_2O): δ 1.38-1.80 (m, 10H), 1.98 (m, 2H), 2.72 (t, 2H, imidazole-CH_2), 3.02 (m, 2H, CH_2NH), 3.22 (m, 1H, NHCH), 4.50 (s, 4H, 4 × CHOH), 7.20 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

Cyclohexyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine tartaric acid salt (1i)

Yield 0.31 g (82%). Yellow wax. ^{1}H NMR (D_2O): δ 1.00-2.10 (m, 14H), 2.78 (t, 3H, imidazole-CH_2), 3.05 (m, 3H, CH_2NH + NHCH), 4.45 (s, 4H, 4 × CHOH), 7.20 (s, 1H, imidazole-5H), 8.55 (s, 1H, imidazole-2H).

Cycloheptyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine tartaric acid salt (1j)

Yield 0.16 g (95%). Yellow wax. ^{1}H NMR (D_2O): δ 1.38-2.18 (m, 16H), 2.75 (t, 2H, imidazole-CH_2), 3.05 (m, 2H, CH_2-NH), 3.28 (m, 1H, CHNH), 4.48 (s, 4H, 4 × CHOH), 7.20 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

Benzyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine oxalic acid salt (1k)

Yield 0.78 g (48%). White powder. Mp: 232-234 °C (d). ^{1}H NMR (D_2O): δ 1.65-1.80 (m, 4H, central CH_2's), 2.76 (t, 2H, imidazole-CH_2), 3.09 (m, 2H, CH_2NH), 4.20 (s, 2H, CH_2-phenyl), 7.20 (s, 1H, imidazole-5H), 7.48 (m, 5H, phenyl-H), 8.57 (s, 1H, imidazole-2H).

4-Chloro-benzyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine oxalic acid salt (1l)

Yield 0.058 g (51%). White powder. Mp: 194.5-196.5 °C. ^{1}H NMR (D_2O): δ 1.68 (m, 4H, central CH_2's), 2.70 (t, 2H, imidazole-CH_2), 3.00 (m, 2H, CH_2NH), 4.18 (s, 2H, CH_2-phenyl), 7.15 (s, 1H, imidazole-5H), 7.31-7.50 (m, 4H, phenyl-H), 8.52 (s, 1H, imidazole-2H).

[4-(1*H*-imidazol-4-yl)-butyl]-dimethyl-amine tartaric acid salt (1m)

Yield 0.089 g (35%). Yellow wax. ^{1}H NMR (D_2O): δ 1.72 (m, 4H, central CH_2's), 2.70-2.95 (m, 8H, $N(CH_3)_2$ + imidazole-CH_2), 3.12 (t, 2H, CH_2NH), 4.45 (s, 4H, 4 × CHOH), 7.18 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

[4-(1*H*-imidazol-4-yl)-butyl]-diethyl-amine oxalic acid salt (1n)

Yield 0.34 g (49%). White powder. Mp: 135-135.5 °C. ^{1}H NMR (D_2O): δ 1.20 (t, 6H, $(CH_3CH_2)_2$), 1.55-1.80 (m, 4H central CH_2's), 2.70 (t, 2H, imidazole-CH_2), 2.95-3.22 (m, 6H, $CH_2N(CH_2)_2$), 7.18 (s, 1H, imidazole-4H), 8.50 (s, 1H, imidazole-2H).

4-(4-Pyrrolidin-1-yl-butyl)-1*H*-imidazole tartaric acid salt (1o)

Yield 0.14 g (24%). Yellow wax. ^{1}H NMR (D_2O): δ 1.60-1.78 (m, 4H, 2 × CH_2), 1.88-2-20 (m, 4H, 2 × CH_2), 2.70 (t, 2H, imidazole-CH_2), 3.00 (m, 2H, 1 × CH_2), 3.18 (m, 2H, 1 × CH_2), 3.58 (m, 2H, 1 × CH_2), 4.41 (s, 4H, 4 × CHOH), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

1-[4-(1*H*-Imidazol-4-yl)-butyl]-piperidine tartaric acid salt (1p)

Yield 0.38 g (45%). Yellow wax. ^{1}H NMR (D_2O): δ 1.28-2.00 (m, 10H), 2.58-2.95 (m, 4H), 3.05 (m, 2H), 3.45 (m, 2H), 4.40 (s, 4H, 4 × CHOH), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

1-[4-(1*H*-Imidazol-4-yl)-butyl]-azepane tartaric acid salt (1q)

Yield 0.092 g (34%). Yellow wax. ^{1}H NMR (D_2O): δ 1.50-1.95 (m, 12H), 2.72 (t, 2H, imidazole-CH_2), 3.10 (m, 4H), 3.25-3.48 (m, 2H), 4.45 (s, 4H, 4 × C*H*OH), 7.18 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

Ethyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine oxalic acid salt (2b)

Yield 0.26 g (30%). White powder. Mp: 141.8-143.5 °C. ^{1}H NMR (D_2O): δ 1.25 (t, 3H, CH_3CH_2), 1.38 (m, 2H, 1 × CH_2), 1.50-1.85 (m, 4H, 2 × CH_2), 2.68 (m, 2H, imidazole-CH_2), 2.85-3.10 (m, 4H, C*H*2NHCH_2), 7.15 (s, 1H, imidazole-5H), 8.50 (s, imidazole-2H).

[5-(1*H*-Imidazol-4-yl)-pentyl]-propyl-amine oxalic acid salt (2c)

Yield 0.19 g (46%). Light beige powder. Mp: 151-152 °C. ^{1}H NMR (D_2O): δ 0.92 (t, 3H $CH_3CH_2CH_2$), 1.30-1.80 (m, 8H, $CH_3CH_2CH_2$ + central CH_2's), 2.70 (t, 2H, imidazole-CH_2), 2.95 (m, 4H, CH_2NHCH_2), 7.15 (imidazole-5H), 8.50 (s, 1H, imidazole-2H).

[5-(1*H*-imidazol-4-yl)-pentyl]-isopropyl-amine oxalic acid salt (2d)

Yield 0.20 g (17%). White powder. Mp: 139.9-143.9 °C. ^{1}H NMR (D_2O): δ 1.25 (d, 6H, $(CH_3)_2CH$), 1.40 (m, 2H, 1 × CH_2), 1.55-1.78 (m, 4H, 2 × CH_2), 2.70 (t, 2H, imidazole-CH_2), 2.98 (m, 2H, CH_2NH), 3.35 (m, 1H, $CH(CH_3)_2$), 7.18 (s, 1H, imidazole-5H), 8.02 (s, 1H, imidazole-2H).

Butyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine oxalic acid salt (2e)

Yield 0.38 g (63%). White powder. Mp: 156-157 °C. ^{1}H NMR (D_2O): δ 0.85 (t, 3H, CH_3), 1.30 (m, 4H, 2 × CH_2), 1.50-1.75 (m, 6H, 3 × CH_2), 2.65 (m, 2H, imidazole-CH_2), 2.95 (m, 4H, CH_2NHCH_2), 7.13 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

[5-(1*H*-imidazol-4-yl)-pentyl]-isobutyl-amine dihydrochloride (2f)

Yield 0.33 g (69%). Beige powder. Mp: 168-170.5 °C. ^{1}H NMR (D_2O): δ 0.92 (d, 6H, $(CH_3)_2CH$), 1.35 (m, 2H, 1 × CH_2), 1.65 (m, 4H, 2 × CH_2), 1.95 (m, 1H, $CH(CH_3)_2$), 2.68 (t, 2H, imidazole-CH_2), 2.83 (d, 2H, $CHCH_2NH$), 2.98 (t, 2H, CH_2NH), 7.15 (s, 1H, imidazole-5H), 8.50 (m, 1H, imidazole-2H).

***tert*-Butyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine dihydrochloride (2g)**

Yield 0.19 g (60%). Yellow powder. Mp: 158-161 °C. ^{1}H NMR (D_2O): δ 1.30 (s, 9H, $(CH_3)_3C$), 1.40 (m, 2H, 1 × CH_2), 1.65 (m, 4H, 2 × CH_2), 2.70 (t, 2H, imidazole-CH_2), 2.98 (t, 2H, CH_2NH), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

Cyclopentyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine dihydrochloride (2h)

Yield 0.57 g (62%). White powder. Mp: 144-145 °C. ^{1}H NMR (D_2O): δ 1.20-1.45 (m, 2H), 1.48-1.80 (m, 10H), 1.90-2.15 (m, 2H), 2.68 (t, 2H, imidazole-CH_2), 2.95 (m, 2H, CH_2NH), 3.50 (m, 1H, NHC*H*), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

Cyclohexyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine dihydrobromide (2i)

Yield 0.50 g (22%). Yellow wax. ^{1}H NMR (D_2O): δ 1.00-2.10 (m, 16H), 2.55 (t, 3H, imidazole-CH_2), 2.95 (m, 3H, CH_2NH + NHC*H*), 6.90 (s, 1H, imidazole-5H), 7.79 (s, 1H, imidazole-2H).

Cycloheptyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine dihydrobromide (2j)

Yield 0.45 g (61%). Beige powder. Mp: 115-117 °C. ^{1}H NMR (D_2O): δ 1.30-2.18 (m, 18H), 2.70 (t, 2H, imidazole-CH_2), 3.02 (m, 2H, CH_2-NH), 3.28 (m, 1H, C*H*NH), 7.20 (s, 1H, imidazole-5H), 8.55 (s, 1H, imidazole-2H).

Benzyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine oxalic acid salt (2k)

Yield 0.56 g (46%). White powder. Mp: 232-234 °C (d). ^{1}H NMR (D_2O): δ 1.29-1.50 (m, 2H, 1 × CH_2), 1.58-1.80 (m, 4H, 2 × CH_2), 2.72 (t, 2H, imidazole-CH_2), 3.05 (m, 2H, CH_2NH), 4.20 (s, 2H, CH_2-phenyl), 7.18 (s, 1H, imidazole-5H), 7.50 (m, 5H, phenyl-H), 8.55 (s, 1H, imidazole-2H).

4-Chloro-benzyl-[5-(1*H*-imidazol-4-yl)-pentyl]-amine oxalic acid salt (2l)

Yield 0.41 g (82%). Yellow powder. Mp: 184.5-186.5 °C. ^{1}H NMR (D_2O): δ 1.35-1.53 (m, 2H, 1 × CH_2), 1.58-1.85 (m, 4H, 2 × CH_2), 2.70 (t, 2H, imidazole-CH_2), 3.05 (t, 2H, CH_2NH), 4.20 (s, 2H, CH_2-phenyl), 7.18 (s, 1H, imidazole-5H), 7.33-7.55 (m, 4H, phenyl-H), 8.52 (s, 1H, imidazole-2H).

[5-(1*H*-imidazol-4-yl)-pentyl]-dimethyl-amine oxalic acid salt (2m)

Yield 0.55 g (85%). Beige powder. Mp: 102.5-103.5 °C. ^{1}H NMR (D_2O): δ 1.23-1.80 (m, 6H, central CH_2's), 2.70 (t, 2H, imidazole-CH_2), 2.82 (s, 6H, N(CH_3)$_2$), 3.07 (t, 2H, CH_2NH), 7.15 (s, 1H, imidazole-5H), 8.48 (s, 1H, imidazole-2H).

[5-(1*H*-imidazol-4-yl)-pentyl]-diethyl-amine oxalic acid salt (2n)

Yield 0.43 g (40%). Yellow powder. Mp: 60.5-62 °C. ^{1}H NMR (D_2O): δ 1.22 (t, 6H, (CH_3CH_2)$_2$), 1.38 (m, 2H, 1 × CH_2), 1.55-1.78 (m, 4H, 2 × CH_2), 2.70 (t, 2H, imidazole-CH_2), 2.95-3.25 (m, 6H, CH_2N(CH_2)$_2$), 7.15 (s, 1H, imidazole-4H), 8.50 (s, 1H, imidazole-2H).

4-(5-Pyrrolidin-1-yl-pentyl)-1*H*-imidazole tartaric acid salt (2o)

Yield 0.18 g (34%). Yellow powder. Mp: 79-81 °C. ^{1}H NMR (D_2O): δ 1.28-1.48 (m, 2H, 1 × C*H*$_2$), 1.58-1.81 (m, 4H, 2 × C*H*$_2$), 1.88-2-20 (m, 4H, 2 × C*H*$_2$), 2.70 (t, 2H, imidazole-C*H*$_2$), 2.90-3.20 (m, 4H, 2 × C*H*$_2$), 3.60 (m, 2H, 1 × C*H*$_2$), 3.58 (m, 2H, 1 × C*H*$_2$), 4.40 (s, 4H, 4 × C*H*OH), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

1-[5-(1*H*-Imidazol-4-yl)-pentyl]-piperidine oxalic acid salt (2p)

Yield 0.43 g (95%). Yellow wax. ^{1}H NMR (D_2O): δ 1.28-1.95 (m, 12H), 2.60-3.10 (m, 6H), 3.45 (m, 2H), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

1-[5-(1*H*-Imidazol-4-yl)-pentyl]-azepane tartaric acid salt (2q)

Yield 1.05 g (74%). Brown wax. ^{1}H NMR (D_2O): δ 1.30-1.50 (m, 2H), 1.60-2.00 (m, 12H), 2.72 (t, 2H, imidazole-CH_2), 3.02-3.28 (m, 4H), 3.32-3.52 (m, 2H), 4.50 (s, 4H, 4 × C*H*OH), 7.15 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

[6-(1*H*-imidazol-4-yl)-hexyl]-isopropyl-amine oxalic acid salt (3d)

Yield 1.18 g (26%). White powder. Mp: 82-83.5 °C. ^{1}H NMR (D_2O): δ 1.28 (d, 6H, (C*H*$_3$)$_2$CH), 1.32 (m, 2H, 1 × C*H*$_2$), 1.60 (m, 4H, 2 × C*H*$_2$), 2.20 (m, 2H, 1 × C*H*$_2$), 2.68 (t, 2H, imidazole-C*H*$_2$), 2.98 (m, 2H, C*H*$_2$NH), 3.35 (m, 1H, C*H*(CH$_3$)$_2$), 7.13 (s, 1H, imidazole-5H), 8.50 (s, 1H, imidazole-2H).

Cyclohexyl-[6-(1*H*-imidazol-4-yl)-hexyl]-amine dihydrochloride (3i)

Yield 1.62 g (54%). Brown powder. Mp: 102-104 °C. ^{1}H NMR (D_2O): δ 1.00-2.10 (m, 16H), 2.68 (m, 4H, imidazole-CH_2 + 1 × C*H*$_2$), 3.00 (m, 3H, C*H*$_2$NH + NHC*H*), 7.18 (s, 1H, imidazole-5H), 8.52 (s, 1H, imidazole-2H).

Benzyl-[6-(1*H*-imidazol-4-yl)-hexyl]-amine oxalic acid salt (3k)

Yield 0.59 g (40%). Beige powder. Mp: 140.4-142 °C. ^{1}H NMR (D_2O): δ 1.25-1.48 (m, 4H, 2 × C*H*$_2$), 1.52-1.78 (m, 4H, 2 × C*H*$_2$), 2.68 (t, 2H, imidazole-C*H*$_2$), 3.03 (m, 2H, C*H*$_2$NH), 4.20 (s, 2H, CH$_2$-phenyl), 7.15 (s, 1H, imidazole-5H), 7.42 (m, 5H, phenyl-H), 8.52 (s, 1H, imidazole-2H).

Acknowledgments

The authors thank Dr. T. Lovenberg, Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA, for the cells stably expressing the human histamine H_3 and H_4 receptors.

References

(1) Leurs, R.; Smit, M. J.; Timmerman, H. Molecular Pharmacological Aspects of Histamine Receptors. *Pharmacol. Ther.* **1995**, *66*, 413-463.
(2) Hill, S. J.; Ganellin, C. R.; Timmerman, H.; Schwartz, J. C.; Shankley, N. P.; Young, J. M.; Schunack, W.; Levi, R.; Haas, H. L. International Union of Pharmacology. XIII. Classification of Histamine Receptors. *Pharmacol. Rev.* **1997**, *49*, 253-278.
(3) Lovenberg, T. W.; Roland, B. L.; Wilson, S. J.; Jiang, X.; Pyati, J.; Huvar, A.; Jackson, M. R.; Erlander, M. G. Cloning and Functional Expression of the Human Histamine H_3 Receptor. *Mol. Pharmacol.* **1999**, *55*, 1101-1107.
(4) Oda, T.; Morikawa, N.; Saito, Y.; Masuho, Y.; Matsumoto, S. I. Molecular Cloning and Characterization of a Novel Type of Histamine Receptor Preferentially Expressed in Leukocytes. *J. Biol. Chem.* **2000**, *275*, 36781-36786.
(5) Arrang, J. M.; Garbag, M.; Schwartz, J. C. Auto-inhibition of Brain Histamine Release Mediated by a Novel Class (H_3) of Histamine Receptors. *Nature Lond.* **1983**, *302*, 832-837.
(6) Lovenberg, T. W.; Pyati, J.; Chang, H.; Wilson, S. J.; Erlander, M. G. Cloning of Rat Histamine H_3 Receptor Reveal Distinct Species Pharmacological Profiles. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 771-778.
(7) Arrang, J. M.; Garbarg, M.; Schwartz, J. C. Autoregulation of Histamine Release in Brain by Presynaptic H_3 Receptors. *Neuroscience* **1985**, *15*, 553-562.
(8) Arrang, J. M.; Garbarg, M.; Schwartz, J. C. Autoinhibition of Histamine Synthesis Mediated by Presynaptic H_3 Receptors. *Neuroscience* **1987**, *23*, 149-157.
(9) Clapham, J.; Kilpatrick, G. J. Histamine H_3 Receptors Modulate the Release of [^{3}H]-Acetylcholine from Slices of Rat Entorhinal Cortex: Evidence for the Possible Existence of H_3 Receptor Sybtypes. *Br. J. Pharmacol.* **1992**, *107*, 919-923.
(10) Yokotani, K.; Murakami, Y.; Okada, S.; Wang, M.; Nakamura, K. Histamine H_3 Receptor-mediated Inhibition of Endogenous Acetylcholine Release from the Isolated, Vascularly Perfused Rat Stomach. *Eur. J. Pharmacol.* **2000**, *392*, 23-29.
(11) Schlicker, E.; Fink, K.; Detzner, M.; Gothert, M. Histamine Inhibits Dopamine Release in the Mouse Striatum via Presynaptic H_3 Receptors. *J. Neural Transm. Gen. Sect.* **1993**, *93*, 1-10.
(12) Schlicker, E.; Schunack, W.; Gothert, M. Histamine H_3 Receptor-mediated Inhibition of Noradrenaline Release in Pig Retina Discs. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1990**, *342*, 497-501.
(13) Schlicker, E.; Betz, R.; Gothert, M. Histamine H_3 Receptor-mediated Inhibition of Serotonin Release in the Rat Brain Cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1988**, *337*, 588-590.
(14) Krause, M.; Stark, H.; Schunack, W. Medicinal Chemistry of Histamine H_3 Receptor Agonists. *The Histamine H_3 Receptor, A Target for New Drugs.*; Elsevier: Amsterdam, 1998.
(15) Onodera, K.; Watanabe, T. Histamine H_3 Antagonists as Potential Therapeutics in the CNS. *The Histamine H_3 Receptor, A Target for New Drugs.*; Elsevier: Amsterdam, 1998.
(16) Leurs, R.; Blandina, P.; Tedford, C.; Timmerman, H. Therapeutic Potential of Histamine H_3 Receptor Agonists and Antagonists. *Trends Pharm. Sci.* **1998**, *19*, 177-183.

(17) Miyazaki, S.; Imaizumi, M.; Onodera, K. Effects of Thioperamide, a Histamine H_3 Antagonist, on a Scopolamine-induced Learning Deficit Using an Elevated Plus-Maze Test in Mice. *Life Sci.* **1995**, *57*, 2137-2144.

(18) Onodera, K.; Miyazaki, S.; Imaizumi, M.; Stark, H.; Schunack, W. Improvement by FUB 181, a Novel Histamine H_3-Receptor Antagonist, of Learning and Memory in the Elevated Plus-maze Test in Mice. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *357*, 508-513.

(19) Passani, M. B.; Cangioli, I.; Bacciottini, L.; Mannaioni, P. F. Thioperamide and Cimetidine Modulate Acetylcholine Release from the Amygdala of Freely Moving Rats. *Inflamm. Res.* **2000**, *49*, S43-44.

(20) Yokoyama, H.; Onodera, K.; Iinuma, K.; Watanabe, T. Effect of Thioperamide, a Histamine H_3 Receptor Antagonist, on Electrically Induced Convulsions in Mice. *Eur. J. Pharmacol.* **1993**, *234*, 129-133.

(21) Yokoyama, H.; Onodera, K.; Maeyama, K.; Sakurai, E.; Iinuma, K.; Leurs, R.; Timmerman, H.; Watanabe, T. Clobenpropit (VUF 9153), a New Histamine H_3 Receptor Antagonists, Inhibits Electrically Induced Convulsions in Mice. *Eur. J. Pharmacol.* **1994**, *260*, 23-28.

(22) Schlicker, E.; Marr, I. The Moderate Affinity of Clozapine at H_3 Receptors is not shared by its two Major Metabolites and by Structurally Related and Unrelated Atypical Neuroleptics. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1996**, *353*, 290-294.

(23) Pillot, C.; Ortiz, J.; Heron, A.; Ridray, S.; Schwartz, J.-C.; Arrang, J. M. Cyproxifan, a Histamine H_3 Antagonist/Inverse Agonist, potentiates Neurochemical and Behavioral Effects of Haloperidol in the Rat. *J. Neurosci.* **2002**, *22*, 7272-7280.

(24) Sakata, T.; Yoshimatsu, H.; Kurokawa, M. Hypothalamic Neuronal Histamine: Implications of its Homeostatic Control of Energy Metabolism. *Nutrition* **1997**, *13*, 403-411.

(25) Takahashi, K.; Suwa, H.; Ishikawa, T.; Kotani, H. Targeted Disruption of H_3 Receptors Results in Changes in Brain Histamine Tone Leading to an Obese Phenotype. *J. Clin. Invest.* **2002**, *110*, 1791-1799.

(26) Bray, G. A.; Tartaglia, L. A. Medicinal Strategies in the Treatment of Obesity. *Nature* **2000**, *404*, 672-677.

(27) Levi, R.; Smith, N. C. Histamine H_3 Receptors: A New Frontier in Myocardial Ischemia. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 825-830.

(28) McLeod, R. L.; Aslanian, R.; Del Prado, M.; Duffy, R.; Egan, R. W.; Kreutner, W.; McQuade, R.; Hey, J. A. Sch 50971, an Orally Active Histamine H_3 Receptor Agonist, Inhibits Central Neurogenic Vascular Inflammation and Produces Sedation in the Guinea Pig. *J. Pharmacol. Exp. Ther.* **1998**, *287*, 43-50.

(29) Bertaccini, G.; Coruzzi, G.; Poli, E. The Histamine H_3 Receptor: a Novel Prejunctional Receptor Regulating Gastro-intestinal Function. *Aliment Pharmacol. Ther.* **1991**, *5*, 585-591.

(30) Mignot, E.; Taheri, S.; Nishino, S. Emerging Therapeutic Targets for Sleep Disorders. *Nature Neurosci.* **2002**, *5*, 1071-1075.

(31) Costa, T.; Ogino, Y.; Munson, P. J.; Onaran, O.; Rodbard, D. Drug Efficacy at Guanine Nucleotide-binding Regulatory Protein-linked Receptors: Thermodynamics Interpretation of Negative Antagonism and of Receptor Activity in the Absence of Ligand. *Mol. Pharmacol.* **1992**, *41*, 549-560.

(32) Lefkowitz, R. L.; Cotecchia, S.; Samama, P.; Costa, T. Constitutive Activity of Receptors Coupled to Guanine Nucleotide Regulatory Proteins. *Trends Pharmacol. Sci.* **1993**, *14*, 303-307.

(33) Milligan, G.; Bond, R. A.; Lee, M. Inverse Agonism: Pharmacological Curiosity or Potential Therapeutic Strategy? *Trends Pharmacol. Sci.* **1995**, *16*, 10-13.

(34) Leurs, R.; Smit, M. J.; Alewijnse, A. E.; Timmerman, H. Agonist-independent Regulation of Constitutively Active G-protein Coupled Receptors. *Trends Biochem. Sci.* **1998**, *23*, 418-422.

(35) Leurs, R.; Rodriguez Pena, M. S.; Bakker, R. A.; Alewijnse, A. E.; Timmerman, H. Constitutive Activity of G-protein Coupled Receptors and Drug Action. *Pharm. Acta Helv.* **2000**, *74*, 327-331.

(36) Milligan, G. Constitutive Activity and Inverse Agonists of G Protein-Coupled Receptors: a Current Perspective. *Mol. Pharmacol.* **2003**, *64*, 1271-1276.

(37) Smit, M. J.; Leurs, R.; Alewijnse, A. E.; Blauw, J.; Van Nieuw Amerongen, G. P.; Van de Vrede, Y.; Roovers, E.; Timmerman, H. Inverse Agonism of Histamine H_2 Antagonists Accounts for Upregulation of Spontaneously Active Histamine H_2 Receptors. *Proc. Natl. Acad. Sci.* **1996**, *93*, 6802-6807.

(38) Bakker, R. A.; Wieland, K.; Timmerman, H.; Leurs, R. Constitutive Activity of the Histamine H_1 Receptor Reveals Inverse Agonism of Histamine H_1 Receptor Antagonists. *Eur. J. Pharmacol.* **2000**, *381*, R5-R7.

(39) Govoni, M.; Bakker, R. A.; van de Wetering, I.; Smit, M. J.; Menge, W. M. B. P.; Timmerman, H.; Elz, S.; Schunack, W.; Leurs, R. Synthesis and Pharmacological Identification of Neutral Histamine H_1-Receptor Antagonists. *J. Med. Chem.* **2003**, *46*, 5812-5824.

(40) Morisset, S.; Rouleau, A.; Ligneau, X.; Gbahou, F.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Schwartz, J. C.; Arrang, J. M. High Constitutive Activity of Native H_3 Receptors Regulates Histamine Neurons in Brain. *Nature* **2000**, *408*, 860-864.

(41) Wieland, K.; Bongers, G.; Yamamoto, Y.; Hashimoto, T.; Yamatodani, A.; Menge, W. M. P. B.; Timmerman, H.; Lovenberg, T. W.; Leurs, R. Constitutive Activity of Histamine H_3 Receptors Stably Expressed in SK-N-MC Cells: Display of Agonism and Inverse Agonism by H_3 Antagonists. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 908-914.

(42) Drutel, G.; Peitsaro, N.; Karlstedt, K.; Wieland, K.; Smit, M. J.; Timmerman, H.; Panula, P.; Leurs, R. Identification of Rat H_3 Receptor Isoforms with Different Brain Expression and Signaling Properties. *Mol. Pharmacol.* **2001**, *1*, 1-8.

(43) Vollinga, R. C.; Menge, W. M. P. B.; Leurs, R.; Timmerman, H. New Analogs of Burimamide as Potent and Selective Histamine H_3 Receptor Antagonists: The Effect of Chain Lenght Variation of the Alkyl Spacer and Modifications of the *N*-Thiourea Substituent. *J. Med. Chem.* **1995**, *38*, 2244-2250.

(44) Vollinga, R. C.; Menge, W. M. P. B.; Leurs, R.; Timmerman, H. Homologs of Histamine as Histamine H_3 Receptor Antagonists: A New Potent and Selective H_3 Antagonist, 4(5)-(5-Aminopentyl)-1*H*-imidazole. *J. Med. Chem.* **1995**, *38*, 266-271.

(45) Ngochindo, R. I. 2-Protecting Groups for 5-Lithiation in the Syntheses of Imidazoles. *J. Chem. Soc., Perkin Trans. 1* **1990**, *6*, 1645-1648.

(46) Mancuso, A. J.; Swern, D. Activated Dimethyl Sulfoxide: Useful Reagents for Synthesis. *Synthesis* **1981**, 165-185.

(47) Omura, K. e. a. Oxidation of Alcohols by "Activated" Dimethyl Sulfoxide. A Preparative, Steric and Mechanistic Study. *Tetrahedron* **1978**, *34*, 1651-1660.

(48) Abdel-Magid, A. F.; Maryanoff, C. A.; Carson, K. G. Reductive Amination of Aldehydes and Ketones by Using Sodium Triacetoxyborohydride. *Tetrahedron Lett.* **1990**, *31*, 5595-5598.

(49) Horne, D. A.; Yakushijin, K.; Büchi, G. A Two-Step Synthesis of Imidazoles From Aldehydes Via 4-Tosyloxazolines. *Heterocycles* **1994**, *39*, 139-153.

(50) Liu, C.; Ma, X. J.; Jiang, X.; Wilson, S. J.; Hofstra, C. L.; Blevitt, J.; Pyati, J.; Li, X.; Chai, W.; Carruthers, N.; Lovenberg, T. W. Cloning and Pharmacological Characterization of a Fourth Histamine Receptor (H_4) Expressed in Bone Marrow. *Mol. Pharmacol.* **2001**, *59*, 420-426.

(51) Kitbunnadaj, R.; Zuiderveld, O. P.; De Esch, I. J. P.; Vollinga, R. C.; Bakker, R. A.; Lutz, M.; Spek, A. L.; Cavoy, E.; Deltent, M.-F.; Menge, W. M. P. B.; Timmerman, H.; Leurs, R. Synthesis and Structure-Activity Relationships of Conformationally Constrained Histamine H_3 Receptor Agonists. *J. Med. Chem.* **2003**, *46*, 5445-5457.

(52) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Anal. Biochem.* **1976**, *72*, 248-254.

(53) De Esch, I. J. P.; Timmerman, H.; Menge, W. M. P. B.; Nederkoorn, P. H. J. A Qualitative Model for the Histamine H_3 Receptor Explaining Agonistic and Antagonistic Activity Simultaneously. *Arch. Pharm. Pharm. Med. Chem.* **2000**, *333*, 254-260.

(54) Uveges, A. J.; Kowal, D.; Zhang, Y.; Spangler, T. B.; Dunlop, J.; Semus, S.; Jones, P. G. The Role of Transmembrane Helix 5 in Agonist Binding to the Human H_3 Receptor. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 451-458.

(55) Van der Werf, J. F.; Bast, A.; Bijloo, G. J.; Van der Vliet, A.; Timmerman, H. HA Autoreceptor Assay with Superfused Slices of Rat Brain Cortex and Electrical Stimulation. *Eur. J. Pharmacol.* **1987**, *138*, 199-206.

(56) Schwartz, J.-C.; Arrang, J.-M.; Garbarg, M.; Schunack, W. *Innovative Approaches in Drug Research*; Elsevier: Amsterdam, 1986; pp 73-89.

(57) Stark, H.; Lipp, R.; Arrang, J.-M.; Garbarg, M.; Schwartz, J.-C.; Schunack, W. Acylated and Alkylated Histamine Derivatives as New Histamine H_3-receptor Antagonists. *Eur. J. Med. Chem.* **1994**, *29*, 695-700.

(58) Topliss, J. G. A Manual Method for Applying the Hansch Approach to Drug Design. *J. Med. Chem.* **1977**, *20*, 463-469.

(59) Van der Goot, H.; Schepers, M. J. P.; Sterk, G. J.; Timmerman, H. Isothiourea Analogues of Histamine as Potent Agonists or Antagonists of the Histamine H_3-Receptor. *Eur. J. Med. Chem.* **1992**, *27*, 511-517.

(60) Arrang, J. M.; Garbag, M.; Lancelot, J. C.; Lecomte, J. M.; Pollard, H.; Robba, M.; Schunack, W.; Schwartz, J. C. Highly Potent and Selective Ligands for Histamine H_3-Receptors. *Nature* **1987**, *327*, 117-123.

(61) Leurs, R.; Kathmann, M.; Vollinga, R. C.; Menge, W. M. B. P.; Schlicker, E.; Timmerman, H. Histamine Homologues Discriminating between Two Functional H_3 Receptor Assay. Evidence for H_3 Receptor Heterogeneity? *J. Pharm. Exp. Ther.* **1996**, *276*, 1009-1015.

(62) Fox, G. B.; Bao Pan, J.; Esbenshade, T. A.; Bennani, Y. L.; Black, L. A.; Faghih, R.; Hancock, A. A.; Decker, M. W. Effects of Histamine H_3 Receptor Ligands GT-2331 and Cyproxifan in a Repeated Acquisition Avoidance Response in the Spontaneously Hypertensive Rat Pup. *Behav. Brain Res.* **2002**, *131*, 151-161.

(63) Tedford, C. E.; Phillips, J. G.; Gregory, R.; Pawlowski, G. P.; Fadnis, L.; Khan, A.; Ali, S. M.; Handley, M. K.; Yates, S. L. Development of *trans*-2-[1*H*-Imidazol-4-yl] Cyclopropane Derivatives as New High-Affinity Histamine H_3 Receptor Ligands. *J. Pharm. Exp. Ther.* **1999**, *289*, 1160-1168.

(64) Gbahou, F.; Rouleau, A.; Morisset, S.; Parmentier, R.; Crochet, S.; Lin, J.-S.; Ligneau, X.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Schwartz, J.-C.; Arrang, J.-M. Protean Agonism at Histamine H_3 Receptors *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci.* **2003**, *100*, 11086-11091.

(65) Nakamura, T.; Itadani, H.; Hikada, Y.; Ohta, M.; Tanaka, K. Molecular Cloning and Characterization of a New Histamine Receptor, *HH4R*. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 615-620.

(66) Morse, K. L.; Behan, J.; Laz, T. M.; West, R. E. J.; Greenfeder, S. A.; Anthes, J. C.; Umland, S.; Wan, Y.; Hipkin, R. W.; Gonsiorek, W.; Shin, N.; Gustafson, E. L.; Qiao, X.; Wang, S.; Hedrick, J. A.; Greene, J.; Bayne, M.; Monsma, F. J. J. Cloning and Characterization of a Novel Human Histamine Receptor. *J. Pharm. Exp. Ther.* **2001**, *296*, 1058-1066.

(67) Nguyen, T.; Shapiro, D. A.; George, S. R.; Setola, V.; Lee, D. K.; Cheng, R.; Rauser, L.; Lee, S. P.; Lynch, K. R.; Roth, B. L.; O'Dowd, B. F. Discovery of a Novel Member of the Histamine Receptor Family. *Mol. Pharmacol.* **2001**, *59*, 427-433.

(68) Zhu, Y.; Michalovich, D.; Wu, H. L.; Tan, K. B.; Dytko, G. M.; Mannan, I. J.; Boyce, R.; Altson, J.; Tierney, L. A.; Li, X.; Herrity, N. C.; Vawter, L.; Sarau, H. M.; Ames, R. S.; Davenport, C. M.; Hieble, J. P.; Wilson, S.; Bergsma, D. J.; Fitzgerald, L. R. Cloning, Expression, and Pharmacological Characterization of a Novel Human Histamine Receptor. *Mol. Pharmacol.* **2001**, *59*, 434-441.

(69) Hough, L. B. Genomics Meets Histamine Receptor: New Subtypes, New Receptors. *Mol. Pharmacol.* **2001**, *59*, 415-419.

(70) Guan, J.; Kyle, D. E.; Gerena, L.; Zhang, Q.; Milhous, W. K.; Lin, A. J. Design, Synthesis, and Evaluation of New Chemosensitizers in Multi-Drug-Resistant *Plasmodium Falciparum*. *J. Med. Chem.* **2002**, *45*, 2741-2748.

(71) Ames, D. E.; Islip, P. J. Syntheses of Long-chain Acids. Part IV. Synthesis of the Octadeca-5,12-dienoic Acids. *J. Chem. Soc.* **1963**, 4363-4368.
(72) Rumbero, A.; Borreguero, I.; Sinisterra, J. V.; Alcantara, A. R. Acyclic Phenylalkanediols as Substrates for the Study of Enzyme Recognition: Synthesis of Substrates and Enzymatic Resolution via Hydrolysis and Transesterification. *Tetrahedron* **1999**, *55*, 14947-14960.
(73) Vollinga, R. C.; Menge, W. M. B. P.; Timmerman, H. A New Convenient Route for the Synthesis of 4(5)-(ω-Aminoalkyl)-1*H*-Imidazoles. *Recl. Trav. Chim. Pays-Bas* **1993**, *112*, 123-125.

Chapter 5

Novel leads for the development of ligands selectively targeting the human histamine H_4 receptor: (*E*)-4-(5-Methyl-1*H*-imidazol-4-yl)-but-3-enylamine and 4-(5-methyl-1*H*-imidazol-4-yl)-butylamine

Marinella Govoni, Herman D. Lim, Iwan J.P. De Esch, Remko A. Bakker,

Henk Timmerman and Rob Leurs

Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry,

Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

Abstract

Insertion of a methyl group in the 5-position of histamine leads to a compound (**1b**) specifically targeting the histamine H_4 receptor (H_4R) over the H_3 receptor (H_3R). In the quest for novel compounds selectively addressing the H_4R, we present here the synthesis and pharmacological investigation of a series of histamine derivatives bearing a longer side chain which may be saturated or unsaturated, as well as the corresponding 5-methyl substituted analogues. The novel ligands were examined for their binding properties and functional activities on both the human H_3R and H_4R subtypes, expressed in SK-N-MC cells. Elongation of the side chain of histamine and/or insertion of an olefinic bond as well as substitution of the primary amino group with a cyanoguanidine moiety generates a series of ligands exhibiting different structure-activity relationships (SAR) on both receptors. Among the 5-methylated compounds, ligands **1d** and **1g** were identified as novel selective H_4R agonists with a 10-fold higher selectivity over the closely related H_3R (**1d**, pK_i values of 7.4 and 6.4; **1g**, pK_i values of 6.5 and 5.5, respectively).

Introduction

The existence of a fourth histamine receptor was postulated by Raible and co-workers as a result of experiments in which histamine produced an increase in cytosolic calcium in eosinophils,[1,2] and confirmed by the recent cloning of the H_4 receptor (H_4R) gene.[3-7] The H_4R is a 390 amino acid, seven-transmembrane G-protein coupled receptor, showing approximately 35% overall homology to the H_3R and a much lower homology to either H_1 or H_2 receptors (23 and 21.6%, respectively).[3] This is reflected in the pharmacological profile of known histamine receptor ligands, where most H_1 and H_2 antagonists do not bind to the H_4R.[8] However, some of the H_3R ligands, e.g. *R*-α-methylhistamine (RAMH) thioperamide, imetit, immepip and clobenpropit (Figure 1), bind to the H_4R, although with affinities different from those for the H_3R.[3-5,8,9]

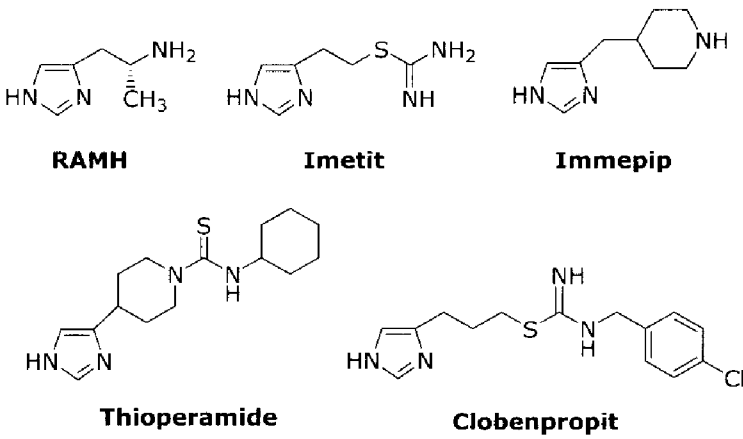
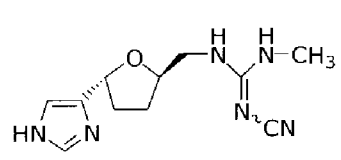


Figure 1. Structures and affinities of histamine ligands *R*-α-methylhistamine (RAMH), thioperamide, imetit, immepip and clobenpropit to the human histamine H_3 and H_4 receptors.[4]

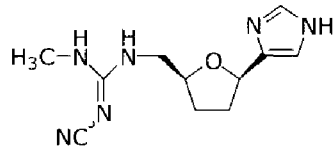
	K_i H_3R (nM)	K_i H_4R (nM)
RAMH	0.7 ± 0.3	146 ± 68
Thioperamide	25 ± 7.4	27 ± 13
Imetit	0.3 ± 0.2	2.7 ± 0.6
Immepip	0.6 ± 0.2	12.8 ± 3.0
Clobenpropit	0.4 ± 0.1	9.0 ± 1.0

There are also great differences in the expression pattern between H_4 and H_3 receptors. The H_3R is mainly expressed in the central nervous system,[10] whereas the mRNA of the H_4R is found mostly in peripheral tissues, such as bone marrow,[4,6] small intestine,[3,4,11] and spleen.[3,6,11] The highest expression of the H_4R is detected in eosinophils and mast cells and indeed the H_4R has been shown to be involved in chemotaxis of both cell types.[1,3,5,12,13] The H_4R has been implicated in the release of IL-16 from $CD8^+$ T cells as well.[14] The physiological and pathophysiological functions of the human H_4R are largely unknown, but its abundant expression in the hematopoietic and lymphatic tissues indicates that the receptor might be involved in the regulation of hematopoiesis and/or an immune function.

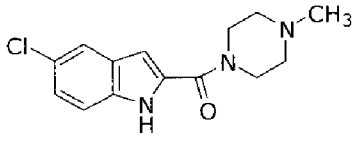
To investigate the possible physiological function of the H_4R and the pharmacology of the receptor, selective ligands are required. To date, only a few relatively selective ligands for the H_4R are available, namely the H_4R agonists (-)-2-cyano-1-methyl-3-{(2*R*,5*R*)-5-[1*H*-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine (OUP-**16**), its (2*R*,5*S*) analogue (Figure 2), respectively showing 41- and 45-fold higher potency at the H_4R than at the H_3R,[15] the non-imidazole H_4R antagonist JNJ 7777120[16,17] and its benzimidazole analogue VUF 6002[18] (Figure 2), displaying correspondingly a 100- and 50-fold higher affinity for the H_4R over the H_3R.[18]



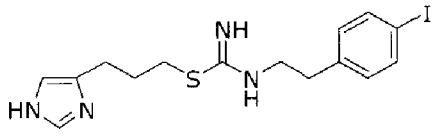
OUP-16



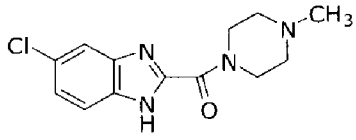
(-)-2-Cyano-1-methyl-3-{(2*R*,5*S*)-5-[1*H*-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine



JNJ 7777120



Iodophenpropit



VUF 6002

Figure 2. A, Structures of the selective H_4R ligands (-)-2-cyano-1-methyl-3-{(2*R*,5*R*)-5-[1*H*-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine (OUP-**16**) and its (2*R*,5*S*) analogue, the potent and selective neutral H_4R antagonist (1-[5-Chloro-1*H*-indol-2-yl)-carbonyl]-4-methylpiperazine (JNJ 7777120), and the neutral H_4R antagonists (5-Chloro-1*H*-benzoimidazol-2-yl)-(4-methyl-piperazin-1-yl)-methanone (VUF 6002) and 2-[3-(1*H*-imidazol-4-yl)-propyl]-1-[2-(4-iodo-phenyl)-ethyl]-isothiourea (iodophenpropit).

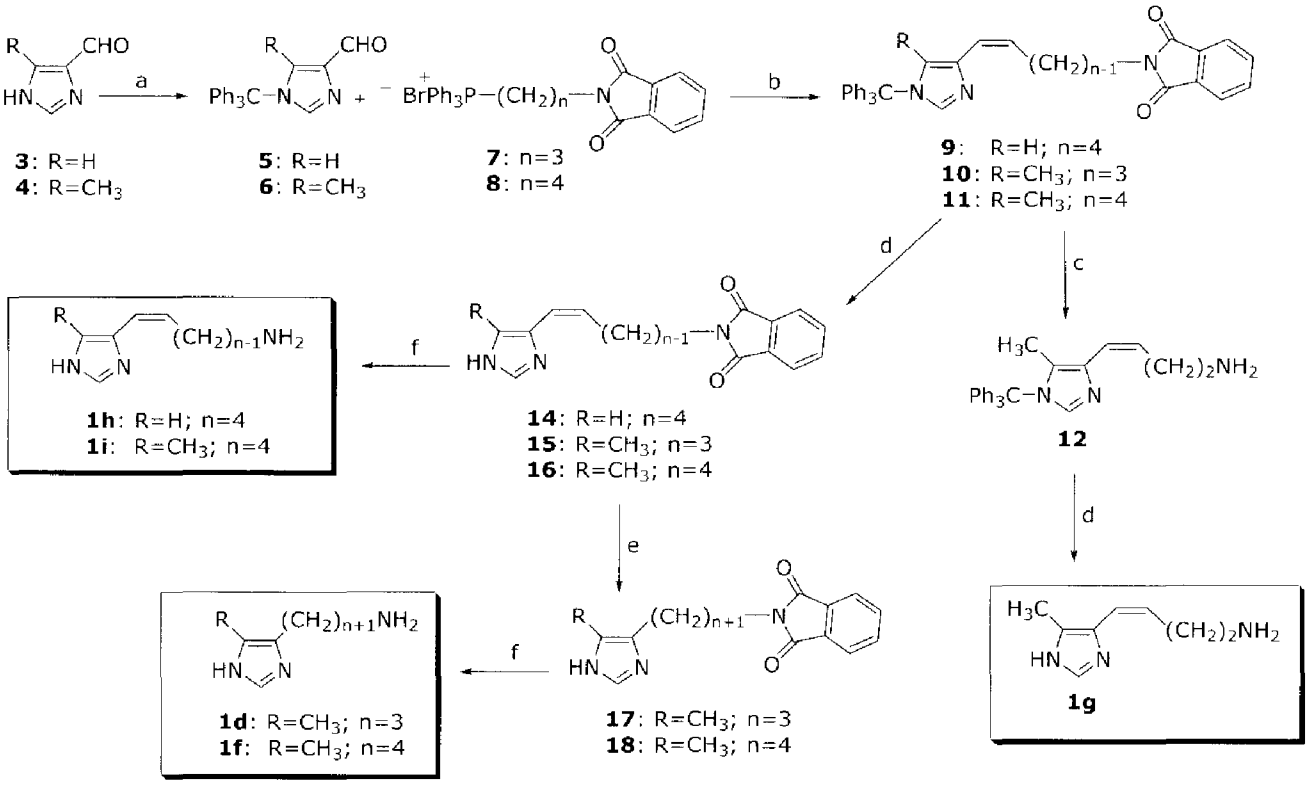
In a preliminary unpublished study from our laboratory, histamine (**1a**) and its 5-methylated analogue **1b** (5-methylhistamine, Figure 3), showed a relatively high affinity and full agonist potency on the human H_4R (Table 1). Yet, when the same compounds were pharmacologically characterized on the human H_3R it was found that the insertion of the methyl group in the 5-position of the imidazole ring of histamine, affording **1b**, is detrimental for the H_3R affinity (pk_i values of 5.2 and 7.6 respectively on the H_3R and H_4R for **1b**, compared to 8.0 and 7.9 for **1a**), hence leading to a molecule with a 125-fold selectivity for the H_4R over the H_3R (**1b**, Table 1 and Figure 4). 5-Methylhistamine (**1b**) is already a well-known ligand for the histamine receptors. In fact, it had been identified in the past as the first agonist that had any selectivity for the histamine H_2 receptor (H_2R),[19] although more potent and selective H_2R agonists are now available.[20]

In view of the fact that the 5-position of the imidazole ring of histamine ligands might be a key position for obtaining compounds discriminating between the H_3R and the H_4R and in the quest for novel ligands selectively targeting the H_4R, we here report the synthesis, affinity and

functional activity on the human H_3R and H_4R of a series of ω-aminoalkenyl- and ω-aminoalkyl-imidazoles, together with the corresponding 5-methyl substituted analogues (Figure 5).

Chemistry

A concise and high yielding 4 or 5 step synthetic sequence was employed to prepare the target ω-aminoalkyl or ω-aminoalkenyl-imidazoles **1d,f,g-i** depicted in Scheme 1 and Figure 5.



Scheme 1. Synthesis of compounds **1d,f,g-i**. Reagents: (a) triphenylmethyl chloride, triethylamine, DMF, r.t.; (b) *t*-BuOK, THF, 0 °C to reflux; (c) hydrazine monohydrate, EtOH, reflux; (d) HCl 1M, EtOH, reflux; (e) 10% Pd/C, MeOH, H_2, 5-10 atm; (f) HBr 30%, reflux.

Treatment of imidazole aldehydes **3** or **4** with triphenylmethyl chloride afforded protected imidazoles **5**[21] or **6**, which were coupled to the phosphonium bromide salts **7**[22] or **8**[22] to give the Wittig olefination products **9-11** in moderate to good yields (37-67%). Hydrazinolysis of the ω-phthalimide group of **10** gave amine **12**, which acidic deprotection afforded compound **1g** in 39% yield. Alternatively, the trityl compounds **9-11** were treated with hydrochloric acid to give deprotected imidazoles **13-15** in excellent yields (85-92%). Hydrogenation of the olefinic bond of **14** and **15** with 10% Pd-C, followed by liberation of the phthalimide moiety with hydrobromic acid provided the ω-aminoalkylimidazoles **1d,f** in high yields (85 and 91%).

The same treatment with hydrobromic acid caused removal of the phthalimide group from compounds **13** and **15**, affording ω-aminoalkenylimidazoles **1h,i** (62 and 29% yield, respectively).

Pharmacology

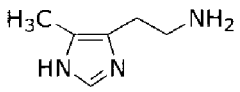
Radioligand Displacement Studies. Homogenates of SK-N-MC cells, stably expressing either the human H_3R[10] or the human H_4R,[4] were used for determining ligand affinities for the H_3R and H_4R respectively, as previously described.[23,24] Cell homogenates of H_3R-expressing cells (475 ± 32 fmol/mg of protein) were incubated for 40 min at 25 °C with 0.9-1.1 nM [^{3}H]-*N*$^\alpha$-methylhistamine (82 Ci/mmol) in 50 mM sodium phosphate buffer (pH 7.4) with or without competing ligands; cell homogenates of H_4R-expressing cells (620 ± 44 fmol/mg of protein) were incubated for 60 min at 37 °C with 9-11 nM [^{3}H]histamine (23.2 Ci/mmol) in 50 mM Tris HCl (pH 7.4), with or without competing ligands. Incubations were terminated by rapid dilution and subsequent filtration over Whatman GF/C filters pretreated with 0.3% polyethyleneimine using ice-cold wash buffer (H_3R: 25mM Tris HCl, 145 mM NaCl, pH 7.4 at 4 °C; H_4R: 50mM Tris HCl, pH 7.4 at 4 °C). The radioactivity retained on the filters was measured by liquid scintillation counting. Nonspecific binding was determined with 1 μM thioperamide as competing ligand. Competition isotherms were evaluated by a nonlinear, least squares curve-fitting using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Colorimetric cAMP Assay. Colorimetric cAMP assays were performed as previously described.[23,24] Briefly, SK-N-MC cells stably expressing the human H_3R[10] or the human H_4R,[4] as well as a cyclic AMP responsive element (CRE)-β-galactosidase reporter-gene were grown overnight in 96-well plates before the assay. To start the assay, the cells were incubated for 6 h with 1 μM forskolin and respective ligands at 37 °C. Thereafter, the medium was aspirated and cells were incubated overnight at 4 °C with 100 μL of assay buffer [100 mM NaH_2PO_4, 100 mM Na_2HPO_4, pH 8, 2 mM $MgSO_4$, 0.1 mM $MnCl_2$, 0.5% Triton, 40 mM β-mercaptoethanol, and 4 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG)]. The absorbance at 405 nm was determined by using a Victor^2 plate reader (Perkin-Elmer).

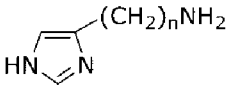
Analytical Methods. Protein levels were determined spectrophotometrically by a Packard Argus 400 microplate reader according to the method of Bradford,[25] using bovine serum albumin as a standard. All data shown are expressed as a mean ± S.E.M., and statistical analyses were carried out by Student's t-test. P values < 0.05 were considered to indicate a significative difference.

Results and discussion

In view of the fact that the 5-position of the imidazole ring of histamine ligands might be a key position for obtaining selective compounds discriminating between the H_3R and the H_4R, the well-known potent H_3R agonist, imetit (**2a**, Figure 3),[26-28] and its 5-methylated analogue (5-methylimetit, **2b**, Figure 3),[26] have been pharmacologically re-characterized using a CRE-mediated β-galactosidase reporter-gene assay (Table 1).

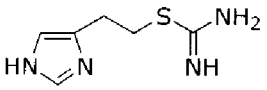


5-Methyl-histamine (1b)

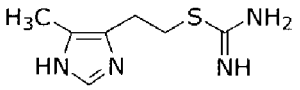


n=4: **Imbutamine (1c)**
n=5: **Impentamine (1e)**

Figure 3. Structures of the discussed compounds. Imetit (**2a**, VUF 8325), 5-methyl-imetit (**2b**, VUF 9032), 5-methyl-histamine (**1b**), imbutamine (**1c**, VUF 4701) and impentamine (**1e**, VUF 4702).



Imetit (2a)



5-Methyl-imetit (2b)

In this assay system, the functional activity of the screened compounds is determined by the inhibition or activation of the forskolin-stimulated (1μM) cAMP production in SK-N-MC cells stably expressing the human H_3R[10] or H_4R.[4] This assay already proved a very useful and sensitive method for the assessment of the functional activity of a number of compounds.[23,24] However, the structure-affinity relationships (SAR) of **2a,b** proved to be different from those of **1a,b**. Insertion of a methyl group in the 5-position of the imidazole ring of **2a** caused a similar decrease in affinity and potency of **2b** on both the H_3 and H_4 receptors, and thus did not lead to a selective H_4R ligand (Figure 4). Both methylated analogues **1b** and **2b** displayed partial H_3R agonism, though with different intrinsic activity values (α=0.64 and 0.82, respectively), but whilst **1b** retained a full agonistic activity on the H_4R, **2b** showed a reduced intrinsic H_4R activity (α=0.59).

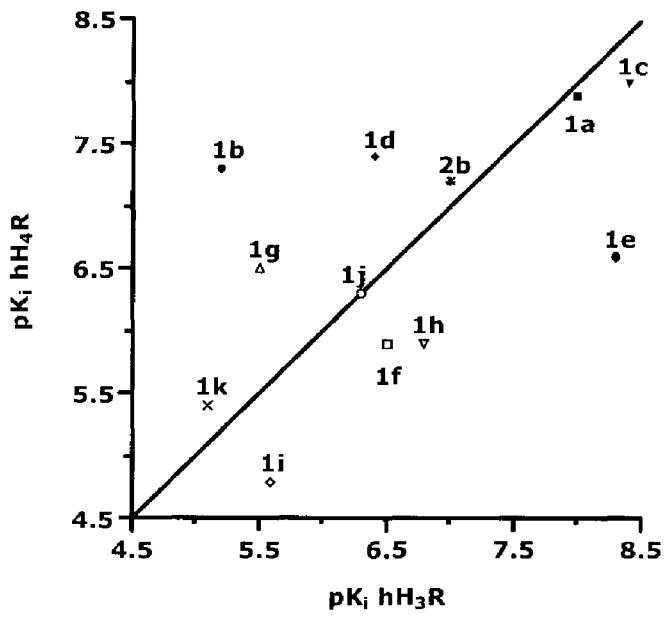


Figure 4. Relation of the affinity between the H_3 and H_4 receptors. Data for compounds in Table 1 are plotted as human H_3R pK_i values (abscissa) versus human H_4R pK_i values (ordinate).

Nevertheless, the relative H_4R/H_3R specificity showed by compound **1b**, prompted us to regard it as a starting point for the development of additional ligands selectively targeting the H_4R, over the closely related H_3R. The generated ligands might as well serve for the establishment of SAR on the H_3 and H_4 receptors. The synthesised compounds are depicted in Figure 5. Some of the ligands have been described before,[29,30] but were synthesised differently in this work. The compounds were pharmacologically investigated on both the H_3R and H_4R and the results are reported in Table 1.

Table 1. Pharmacological properties of compounds **1a-k** and **2a,b** for the human H_3R and H_4R. The values are expressed as means ± SEM of three separate experiments, each performed in triplicate.

	hH_3R			hH_4R		
compd	pK_i	pEC_{50}	α	pK_i	pEC_{50}	α
Histamine (**1a**)	8.0 ± 0.1	8.3 ± 0.1	1	7.9 ± 0.1	7.7 ± 0.1	1
1b	5.2 ± 0.1	5.0 ± 0.1	0.64 ± 0.04	7.3 ± 0.1	7.2 ± 0.1	1.01 ± 0.03
1c	8.4 ± 0.1	8.3 ± 0.1	0.98 ± 0.04	8.0 ± 0.1	7.1 ± 0.1	0.64 ± 0.08
1d	6.4 ± 0.1	6.5 ± 0.1	0.88 ± 0.03	7.4 ± 0.1	6.8 ± 0.1	0.86 ± 0.02
1e	8.3 ± 0.1	8.3 ± 0.1	0.71 ± 0.05	6.6 ± 0.1	<5	-[b]
1f	6.5 ± 0.1	6.5 ± 0.4	0.23 ± 0.04	5.9 ± 0.1	-[b]	0
1g	5.5 ± 0.1	5.4 ± 0.2	0.60 ± 0.15	6.5 ± 0.1	6.3 ± 0.2	0.45 ± 0.09
1h	6.8 ± 0.1	6.6 ± 0.1	0.69 ± 0.08	5.9 ± 0.1	-[b]	0
1i	5.6 ± 0.1	5.4 ± 0.1[a]	-1.07 ± 0.24	4.8 ± 0.1	-[b]	0
1j	6.3 ± 0.1	6.4 ± 0.1[a]	-0.89 ± 0.03	6.3 ± 0.1	6.1 ± 0.1	0.62 ± 0.03
1k	5.1 ± 0.2	<5	-[b]	5.4 ± 0.1	5.3 ± 0.2	0.52 ± 0.04
2a	8.8 ± 0.1	9.7 ± 0.1	0.98 ± 0.04	8.2 ± 0.1	7.9 ± 0.1	0.90 ± 0.01
2b	7.0 ± 0.1	7.4 ± 0.1	0.82 ± 0.06	7.2 ± 0.1	6.9 ± 0.1	0.59 ± 0.03

[a] pIC_{50} value. [b] could not be estimated.

Elongation of the side chain of histamine (**1a**) to four methylene units generated the higher homologue imbutamine (**1c**, Figure 3), which retained affinity and full agonist potency on the H_3R, whilst it showed partial agonism on the H_4R (α=0.64, Table 1). Increasing the side chain by one more methylene unit gave impentamine (**1e**, Figure 3), which affinity and potency on the H_3R were comparable to those of **1a** and **1c**. However, the intrinsic activity on the H_3R was reduced, turning **1e** into a partial agonist (α=0.71). The binding H_4R affinity of **1e** was also decreased 20- to 25-fold compared to **1a** and **1c**, and so was the potency of the compound, which in turn prevented the assessment of its intrinsic activity (Table 1).

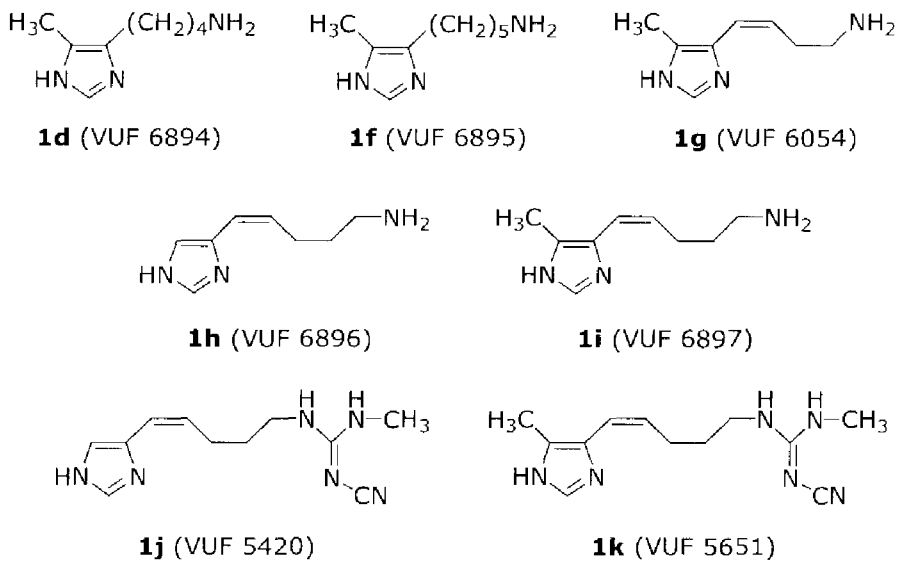
Methylation in the 5-position of the imidazole ring of **1c** and **1e** caused a marked reduction in the H_3R affinity of the afforded compounds, namely 5-methylimbutamine (**1d**) and 5-methylimpentamine (**1f**), as shown in Table 1. Assessment of the pharmacological properties of compound **1d** on the H_4R revealed that, as expected, insertion of the methyl group in the 5-position of imbutamine was better tolerated than in the case of the H_3R, thus leading to a derivative showing a higher affinity for the H_4R than for the H_3R (pk_i values of 7.4 and 6.4, Table 1), even though the H_4 over H_3 selectivity of **1d** was not as high as for **1b** (Figure 4).

Remarkably, in the case of **1f**, 5-methylation did not lead to a ligand showing H_4R over H_3R selectivity (Table 1 and Figure 4).

In a molecular modelling and mutational study on the H_3R, Uveges and coworkers evaluated the activities of histamine, imetit and impentamine on either wild-type or mutant H_3Rs.[31] They demonstrated that the agonist-receptor complexes formed by the three different agonists are not equivalent in their ability to activate the G-protein and suggested that different agonists might thus promote different conformational states of the receptor. The amino acid sequence of the H_4R is approximately 35% identical to the H_3R, but the homology in the transmembrane region rises to 58%,[32] which suggest that the two receptors could possibly have a similar mode of ligand recognition.[4,33] In this light, it is tempting to speculate that those observations could also hold for the H_4R, thus explaining the different H_4R behaviour of **1f** and **1b**.

Moreover, it would also be not surprising that different behaviors in the affinity and functional activity of histamine (**1a**), imetit (**2a**) and their methylated derivatives (**1b,2b**) on the H_3R and H_4R are found in the current study.

We could also hypothesise that the mechanism of interaction and receptor activation by imbutamine (**1c**) and its 5-methyl derivative (**1d**, Figure 5) for both the H_3R and H_4R is comparable to the one of histamine (**1a**) and 5-methylhistamine (**1b**, Figure 3), rather than the one of impentamine (**1e**) and 5-methylimpentamine (**1f**, Figure 5).



1d (VUF 6894) **1f** (VUF 6895) **1g** (VUF 6054)

1h (VUF 6896) **1i** (VUF 6897)

1j (VUF 5420) **1k** (VUF 5651)

Figure 5. Structures of the synthesised compounds **1d,f-k**.

We have previously proposed the existence of multiple binding pockets and a different set of receptor-site points available for interaction with the H_3R for derivatives of **1c** and **1e**, that are very much dependent on the distance between the basic nitrogen of the side chain and the imidazole nucleus as well as on the kind of substituent on the aforementioned basic nitrogen.[24] Interestingly, a lack of intrinsic H_4R activity for compound **1f** was observed, thereby identifying 5-methylimpentamine (**1f**) as a novel neutral H_4R antagonist (Table 1).

So far, only three ligands have been recognized as neutral H_4R antagonists: the potent and selective H_4R ligand JNJ 7777120, previously reported as an antagonists on the H_4R,[16] its benzimidazole analogue (VUF 6002)[18] and the inverse H_3R agonist iodophenpropit (unpublished results) (Figure 2).

In the paper of Uveges *et al.*,[31] it was also proposed that impentamine (**1e**), on interaction with the wild-type H_3R, may form an agonist-receptor complex of lower intrinsic potency compared to the one formed from other full agonists (e.g. histamine). A weaker agonist-receptor complex could thus explain why **1e** is found to behave only as a partial agonist on the H_3R (α=0.71, Table 1), as well as the even lower intrinsic H_3R activity of its 5-methyl analogue (**1f**, α=0.23, Table 1).

The olefinic derivatives of imbutamine and impentamine, as well as their methylated analogues (compounds **1g-i**, Table 1) all showed reduced affinities, potencies and intrinsic activities on both H_3 and H_4 receptors in comparison to their parent compounds **1c,f** (Figure 4). Nevertheless, different SAR depending on the length of the side chain could again be observed. In particular, the methylated olefinic derivative of imbutamine (**1g**, Table 1), showing 10-fold selectivity, was found as an additional compound selectively targeting the H_4R over the H_3R (Figure 4).

On the other hand, olefinic derivatives of impentamine (**1h,i**, Table 1) showed a better selectivity for H_3R over H_4R, as was also observed for their parent compounds **1e,f** (Figure 4). Strikingly, 5-methylation of **1h**, yielding compound **1i** (Table 1), caused an inversion of intrinsic H_3R activity, switching it from partial agonism (**1h**, α=0.69, Table 1) to full inverse agonism (**1i**, α=-1.07, Table 1). Both compounds behaved however as neutral H_4R antagonists.

Substitution of the amino group of the tetrahydrofuranylimidazole imifuramine with a cyanoguanidine moiety yielding compound OUP-16 (Figure 2) led to a decrease in the agonistic H_3R activity and an increase in the H_4R versus H_3R selectivity, thus generating a compound with a 17-fold higher affinity and a 41-fold higher potency at the H_4R than at the H_3R.[15]

However, introduction of a cyanoguanidine moiety on the ω-amino function of imbutamine (**1d**) did not lead to a compound showing higher affinity or potency on the H_4R rather than on the H_3R (**1j**, Table 1). Moreover, the introduced modification caused a reduction in affinity of **1j** on both the H_3R and H_4R, paralleling observations made for histamine, in which replacement of the primary amine by a guanidine moiety is tolerated on the H_3R, but leads to partial agonism.[26] In the case of **1j**, however, an inversion in intrinsic H_3R activity was observed, **1j** being a partial inverse H_3R agonist (α=-0.89, Table 1).

Noticeably, 5-methylation of **1j**, yielding **1k** (Table 1), does lead to a decrease in affinity for both H_3 and H_4 receptors, as already observed in the case of 5-methylation of all the other compounds of this series (**1d,f,g,i**), but does not afford a selective H_4R ligand, as was seen with the other imbutamine related compounds of this series (**1d,g**) (Figure 4). This seems to indicate that, despite the same side chain length, the ligand-receptor complexes formed by **1j** and **1k** are not equivalent to those formed by imbutamine and its related compound (**1c,d,g**), either regarding the conformational states of the H_3R or H_4R, or the intrinsic potency of the formed complex.

Conclusions

Following a study showing a 125-fold selectivity for the H_4R over H_3R of 5-methylhistamine (1b) and in the quest for novel compounds selectively targeting the H_4R, the synthesis of a series of histamine derivatives bearing a longer and/or unsaturated side chain as well as the corresponding 5-methyl substituted analogues has been reported in this study. The novel ligands were pharmacologically characterized *in vitro* on the human H_3R and H_4R subtypes. The isothiourea analogue of histamine, imetit (2a), and its methylated derivative 2b, were also investigated, but proved to have different SAR from those of 1a,b; in fact 5-methylimetit (2b), did not shown any specificity for the H_4R over the H_3R.

Elongation and/or unsaturation of the side chain of histamine giving compounds 1c,e,h affects in different ways the H_3R and H_4R binding and functional properties of the ligands, depending either on the length of the side chain, or on the presence of an olefinic bond. In particular, both elongation of the side chain of 1a to five methylene units or introduction of unsaturation in this side chain dramatically affects the H_4R affinity and functional activity of 1e,h.

Replacement of the primary amino function of 1c by a cyanoguanidine moiety causes a marked decrease in both affinity and potency on the H_3R and H_4R (1j). Remarkably, this modification also leads to an inversion of H_3R intrinsic activity, turning 1j into an almost full inverse agonist.

Introduction of a methyl group in the 5-position of the imidazole ring of the abovementioned compounds is detrimental for all the synthesised ligands, as highlighted by the decrease in the H_3R and H_4R binding affinities. In all cases the affinity reduction was more marked for the H_3R than for the H_4R. However, only in two cases this led to relatively selective H_4R ligands. Imbutamine derivatives 1d,g were in fact found to show a 10-fold H_4R/H_3R specificity, underscoring the importance of the 5-position of the imidazole ring, but also the dependence of the selective behaviours on the length of the side chain and on the substitution of the primary amine.

Compounds **1d** and **1g** might be useful pharmacological tools and may also serve as leads for the development of H_4R ligands with improved potency and selectivity. Moreover, the series of ligands reported in this study seem to belong to two different subclasses showing different SAR, thus making them valuable compounds for modelling and mutational studies, which will ultimately help in the elucidation and better understanding of the structural requirements for agonism, neutral antagonism and inverse agonism at both the H_3R and H_4R subtypes, as well as for achieving highly selective compounds for any of these receptors.

Experimental procedures

General procedures. Reagents were obtained from commercial suppliers and used without further purification. Solvents used were either AR or HPLC grade. Dry THF was freshly distilled from $LiAlH_4$ and DMF was dried on molecular sieves.

Histamine (**1a**) was obtained from Sigma Research Biochemicals Inc. (Zwijndrecht, The Netherlands). 2-(5-Methyl-1*H*-imidazol-4-yl)-ethylamine (**1b**) was a kind gift of GlaxoSmithKline (Welwyn, Garden City, Hertfordshire, UK).

Compounds **1c,e** and **2a,b** were obtained from our own laboratory stock.[26,34]

Compounds **1j,k** were synthesized according to the procedure of Durant *et al.*[29]

Melting points were measured on an Electrothermal IA 9200 apparatus.

^1H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. Chemical shifts are given in ppm using the residual undeuterated solvent as reference.

Flash chromatography was performed on J.T.Baker Kieselgel 60.
All reactions were performed under an atmosphere of dry nitrogen.

5-Methyl-1-trityl-1*H*-imidazole-4-carbaldehyde (6)

5-Methyl-1*H*-imidazole-4-carbaldehyde **4** (5.95 g, 0.054 mol) and Et_3N (14.98 mL, 0.11 mol) were stirred at room temperature (r.t.) in DMF (35 mL). Triphenylmethyl chloride (15.06 g, 0.054 mol) in DMF (80 mL) was added dropwise and the resulting mixture was stirred at r.t. overnight. The mixture was poured into 600 mL of water, which resulted in a yellow precipitate. This solid was isolated by filtration, washed with water and dissolved in dichloromethane (300 mL). The organic layer was successively washed with water and brine, dried ($MgSO_4$) and evaporated to dryness. The residue was purified by flash chromatography switching gradually the eluent from dichloromethane/ethyl acetate (9/1) into dichloromethane/ethyl acetate (1/1) to afford 11.19 g (59%) of product as a white solid. ^1H NMR ($CDCl_3$): δ 1.80 (t, 3H, C*H*$_3$), 7.00-7.39 (m, 16H, imidazole-2H + Ph_3C), 9.98 (s, 1H, C*H*O).

General procedure for the synthesis of the Wittig olefination products 9-11

1 equivalent of the phosphonium salt ([3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propyl]-triphenyl-phosphonium bromide **7**[22] or [4-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-butyl]-triphenyl-phosphonium bromide **8**[22]) was suspended in THF and 1 equivalent of the appropriate aldehyde (1-trityl-1*H*-imidazole-4-carbaldehyde **5**[21] or 5-Methyl-1-trityl-1*H*-imidazole-4-carbaldehyde **6**) was added. The reaction was cooled to 0 °C and potassium *tert*-butoxide (1.2 equivalents) was slowly added. The reaction was stirred at 0 °C for 15 min, allowed to warm to r.t. and then refluxed for 3 h. The reaction mixture was cooled to r.t. and poured into a mixture of diethyl ether, hexane and water (2:1:2). The layers were separated and the organic phase washed with water, brine and dried ($MgSO_4$). The solvent was removed in vacuo and the residue purified either by flash chromatography in dichloromethane/ethyl acetate (9/1) or by washings with diethyl ether.

(*E*)-2-[5-(1-Trityl-1*H*-imidazol-4-yl)-pent-4-enyl]-isoindole-1,3-dione (9)

Yield 5.83 g (67%). ^1H NMR ($CDCl_3$): δ 1.79 (m, 2H, central C*H*$_2$), 2.50 (m, 2H, C*H*$_2$CH=CH), 3.65 (t, 2H, C*H*$_2$-isoindole-1,3-dione), 5.51 (m, 1H, CH=C*H*CH$_2$), 6.20 (d, J=11.5 Hz, 1H, C*H*=CHCH$_2$), 6.70 (s, 1H, imidazole-5H), 7.03-7.40 (m, 16H, imidazole-2H + phenyl-H), 7.57-7.80 (m, 4H, isoindole-1,3-dione-H).

(*E*)-2-[4-(5-Methyl-1-trityl-1*H*-imidazol-4-yl)-but-3-enyl]-isoindole-1,3-dione (10)

Yield 2.88 g (59%). ^{1}H NMR ($CDCl_3$): δ 1.36 (s, 3H, CH_3), 3.13 (m, 2H, CH_2CH=CH), 3.82 (t, 2H, CH_2-isoindole-1,3-dione), 5.47 (m, 1H, CH=$CH$$CH_2$), 6.13 (d, J=11.4 Hz, 1H, CH=$CHCH_2$), 6.99-7.35 (m, 16H, imidazole-2H + phenyl-H), 7.58-7.81 (m, 4H, isoindole-1,3-dione-H).

(*E*)-2-[5-(5-Methyl-1-trityl-1*H*-imidazol-4-yl)-pent-4-enyl]-isoindole-1,3-dione (11)

Yield 2.58 g (37%). ^{1}H NMR ($CDCl_3$): δ 1.80 (m, 2H, central CH_2), 2.78 (m, 2H, CH_2CH=CH), 3.70 (t, 2H, CH_2-isoindole-1,3-dione), 5.50 (m, 1H, CH=$CH$$CH_2$), 6. 70 (d, J=11.6 Hz, 1H, CH=$CHCH_2$), 7.05-7.39 (m, 16H, imidazole-2H + phenyl-H), 7.52-7.80 (m, 4H, isoindole-1,3-dione-H).

(*E*)-4-(5-Methyl-1-trityl-1*H*-imidazol-4-yl)-but-3-enylamine (12)

Compound **10** (0.40 g, 0.76 mmol) was dissolved in warm ethanol (10 mL), hydrazine monohydrate (0.044 mL, 0.91 mmol) was added and the solution was refluxed. After 4 h the reaction mixture was cooled to r.t. and filtered. The filtrate was concentrated in vacuo to give **12** as a brown solid (0.31 g), which was used without further purification.

General procedure for the synthesis of compounds 13-15 and the final compound 1g (removal of the trityl group)

1 equivalent of the trityl-protected compound (**9-12**) was dissolved in absolute ethanol, added with 10 equivalents of hydrochloric acid (1N) and heated under reflux. After 3 h the mixture was cooled down, filtered and evaporated to dryness. The residue was purified by washing (under stirring) with portions of diethyl ether.

(*E*)-2-[5-(1*H*-imidazol-4-yl)-pent-4-enyl]-isoindole-1,3-dione hydrochloride (13)

Yield 0.88 g (92%). ^{1}H NMR ($CDCl_3$/CD_3OD): δ 1.69 (m, 2H, central CH_2), 2.12 (m, 2H, CH_2CH=CH), 3.49 (t, 2H, CH_2-isoindole-1,3-dione), 5.80 (m, 1H, CH=$CH$$CH_2$), 6.05 (d, J=11.6 Hz, 1H, CH=$CHCH_2$), 7.08 (s, 1H, imidazole-5H), 7.45-7.70 (m, 4H, isoindole-1,3-dione-H), 8.54 (s, 1H, imidazole-2H).

(*E*)-2-[4-(5-Methyl-1*H*-imidazol-4-yl)-but-3-enyl]-isoindole-1,3-dione hydrochloride (14)

Crystallized from hot ethanol yielding 0.71 g (85%) of white crystals. Mp: 225.0-227.0 °C (d). ^{1}H NMR ($CDCl_3$/CD_3OD): δ 2.00 (s, 3H, CH_3), 2.41 (m, 2H, CH_2CH=CH), 3.64 (t, 2H, CH_2-isoindole-1,3-dione), 5.82 (m, 1H, CH=$CH$$CH_2$), 6.05 (d, J=11.4 Hz, 1H, CH=$CHCH_2$), 7.23 (s, 1H, imidazole-5H), 7.55-7.72 (m, 4H, isoindole-1,3-dione-H), 8.44 (s, 1H, imidazole-2H).

(*E*)-2-[5-(5-methyl-1*H*-imidazol-4-yl)-pent-4-enyl]-isoindole-1,3-dione hydrochloride (15)

Yield 1.32 g (91%). ^{1}H NMR ($CDCl_3/CD_3OD$): δ 1.68 (m, 2H, central CH_2), 1.98-2.13 (m, 5H, CH_3 + CH_2CH=CH), 3.50 (t, 2H, CH_2-isoindole-1,3-dione), 5.81 (m, 1H, CH=$CH$$CH_2$), 6.00 (d, J=11.6 Hz, 1H, CH=$CHCH_2$), 7.52-7.70 (m, 4H, isoindole-1,3-dione-H), 8.46 (s, 1H, imidazole-2H).

(*E*)-4-(5-Methyl-1*H*-imidazol-4-yl)-but-3-enylamine dihydrochloride (1g)

Crystallized from hot isopropanol/ethanol/diethyl ether yielding 0.067 g (39%) of beige powder. Mp: 180.6-182.0 °C. ^{1}H NMR (CD_3OD): δ 2.30 (s, 3H, CH_3), 2.59 (m, 2H, CH_2CH=CH), 3.09 (t, 2H, CH_2-isoindole-1,3-dione), 6.00 (m, 1H, CH=$CH$$CH_2$), 6.42 (d, J=11.6 Hz, 1H, CH=$CHCH_2$), 8.85 (s, 1H, imidazole-2H).

2-[4-(5-methyl-1*H*-imidazol-4-yl)-butyl]-isoindole-1,3-dione hydrochloride (16)

Compound **14** (0.30 g, 0.94 mmol) was dissolved in methanol (50 mL) and 30 mg of 10% Pd/C was added. The suspension was hydrogenated under 5-10 atm of H_2 for 20 h in a stainless steel bomb. The mixture was filtered and the solvent was evaporated yielding 0.29 g (96%) of product. ^{1}H NMR (D_2O): δ 1.58 (m, 4H, central CH_2's), 2.15 (s, 3H, CH_3), 2.62 (t, 2H, imidazole-CH_2), 3.56 (t, 2H, CH_2-isoindole-1,3-dione), 7.60-7.82 (m, 4H, isoindole-1,3-dione-H), 8.31 (s, 1H, imidazole-2H).

2-[5-(5-methyl-1*H*-imidazol-4-yl)-pentyl]-isoindole-1,3-dione hydrochloride (17)

The same procedure as for compound **16** with 0.50 g (1.49 mmol) of **15** and 50 mg of 10% Pd/C gave 0.48 (96%) of product. ^{1}H NMR (D_2O): δ 1.18 (m, 2H, 1 × CH_2), 1.55 (m, 4H, 2 × CH2), 2.11 (s, 3H, CH_3), 2.58 (t, 2H, imidazole-CH_2), 3.54 (t, 2H, CH_2-isoindole-1,3-dione), 7.65-7.85 (m, 4H, isoindole-1,3-dione-H), 8.30 (s, 1H, imidazole-2H).

General procedure for the synthesis of the final compounds 1d,f,i (removal of the isoindole-1,3 dione group)

1 equivalent of the appropriate compound (**15-17**) was mixed with 10 equivalents of hydrobromic acid (30%) and heated under reflux. After 16 h the mixture was allowed to cool to r.t. and concentrated in vacuo. The residue was purified by crystallization from hot ethanol/ethyl acetate.

4-(5-methyl-1*H*-imidazol-4-yl)-butylamine dihydrobromide (1d)

Yield 0.25 g (85%). Brown powder. Mp: 196.0-197.4 °C. ^{1}H NMR (D_2O): δ 1.68 (m, 4H, central CH_2's), 2.29 (s, 3H, CH_3), 2.73 (t, 2H, imidazole-CH_2), 3.02 (t, 2H, CH_2NH_2), 8.45 (s, 1H, imidazole-2H).

5-(5-methyl-1*H*-imidazol-4-yl)-pentylamine dihydrobromide (1f)

Yield 0.43 g (91%). Yellow powder. Mp: 185.7-186.8 °C. ^{1}H NMR (D_2O): δ 1.38 (m, 2H, 1 × C*H*$_2$), 1.66 (m, 4H, 2 × C*H*2), 2.27 (s, 3H, C*H*$_3$), 2.71 (t, 2H, imidazole-C*H*$_2$), 3.00 (t, 2H, C*H*$_2$NH$_2$), 8.44 (s, 1H, imidazole-2H).

(*E*)-5-(5-methyl-1*H*-imidazol-4-yl)-pent-4-enylamine dihydrobromide (1i)

Yield 0.12 g (29%). Yellow powder. Mp: 237.8-239.0 °C (d). ^{1}H NMR (D_2O): δ 1.88 (m, 2H, central C*H*$_2$), 2.25-2.48 (m, 5H, C*H*3 + C*H*2CH=CH), 3.03 (t, 2H, C*H*$_2$NH$_2$), 6.21 (m, 1H, CH=C*H*CH$_2$), 6.42 (d, J=11.8 Hz, 1H, C*H*=CHCH$_2$), 8.48 (s, 1H, imidazole-2H).

(*E*)-5-(1*H*-imidazol-4-yl)-pent-4-enylamine (1h)

The same procedure as for compound **12** was used with 0.88 g (2.77 mmol) of **13** and 0.40 mL (8.32 mmol) of hydrazine monohydrate. The crude product was purified by crystallization from hot ethanol/ethyl acetate to afford 0.26 g (62%) of white crystals. Mp: 153.0-155.0 °C. ^{1}H NMR (D_2O): δ 1.83 (m, 2H, central C*H*$_2$), 1.07 (m, 2H, C*H*2CH=CH), 3.00 (t, 2H, C*H*$_2$NH$_2$), 5.62 (m, 1H, CH=C*H*CH$_2$), 6.39 (d, J=11.7 Hz, 1H, C*H*=CHCH$_2$), 7.18 (s, 1H, imidazole-5H), 7.74 (s, 1H, imidazole-2H).

Acknowledgments

The authors thank Dr. T. Lovenberg, Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA, for the cells stably expressing the human histamine H_3 and H_4 receptors.

References

(1) Raible, D. G.; Lenahan, T.; Fayvilevich, Y.; Kosinski, R.; Schulman, E. S. Pharmacological Characterization of a Novel Histamine Receptor on Human Eosinophils. *Am. J. Respir. Crit. Care Med.* **1994**, *149*, 1506-1511.

(2) Raible, D. G.; Schulman, E. S.; DiMuzio, J.; Cardillo, R.; Post, T. J. Mast Cell Mediators Prostaglandin-D_2 and Histamine Activate Human Eosinophils. *J. Immunology* **1992**, *148*, 3536-3542.

(3) Oda, T.; Morikawa, N.; Saito, Y.; Masuho, Y.; Matsumoto, S. I. Molecular Cloning and Characterization of a Novel Type of Histamine Receptor Preferentially Expressed in Leukocytes. *J. Biol. Chem.* **2000**, *275*, 36781-36786.

(4) Liu, C.; Ma, X. J.; Jiang, X.; Wilson, S. J.; Hofstra, C. L.; Blevitt, J.; Pyati, J.; Li, X.; Chai, W.; Carruthers, N.; Lovenberg, T. W. Cloning and Pharmacological Characterization of a Fourth Histamine Receptor (H_4) Expressed in Bone Marrow. *Mol. Pharmacol.* **2001**, *59*, 420-426.

(5) Morse, K. L.; Behan, J.; Laz, T. M.; West, R. E. J.; Greenfeder, S. A.; Anthes, J. C.; Umland, S.; Wan, Y.; Hipkin, R. W.; Gonsiorek, W.; Shin, N.; Gustafson, E. L.; Qiao, X.;

Wang, S.; Hedrick, J. A.; Greene, J.; Bayne, M.; Monsma, F. J. J. Cloning and Characterization of a Novel Human Histamine Receptor. *J. Pharm. Exp. Ther.* **2001**, *296*, 1058-1066.

(6) Zhu, Y.; Michalovich, D.; Wu, H. L.; Tan, K. B.; Dytko, G. M.; Mannan, I. J.; Boyce, R.; Altson, J.; Tierney, L. A.; Li, X.; Herrity, N. C.; Vawter, L.; Sarau, H. M.; Ames, R. S.; Davenport, C. M.; Hieble, J. P.; Wilson, S.; Bergsma, D. J.; Fitzgerald, L. R. Cloning, Expression, and Pharmacological Characterization of a Novel Human Histamine Receptor. *Mol. Pharmacol.* **2001**, *59*, 434-441.

(7) Hough, L. B. Genomics Meets Histamine Receptor: New Subtypes, New Receptors. *Mol. Pharmacol.* **2001**, *59*, 415-419.

(8) Nakamura, T.; Itadani, H.; Hikada, Y.; Ohta, M.; Tanaka, K. Molecular Cloning and Characterization of a New Histamine Receptor, *HH4R*. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 615-620.

(9) Oda, T.; Matsumoto, S. I.; Masuho, Y.; Takasaki, J.; Matsumoto, M.; Kamohara, M.; Saito, T.; Ohishi, T.; Soga, T.; Hiyama, H.; Matsushime, H.; Furuichi, K. cDNA Cloning and Characterization of Porcine Histamine H_4 Receptor. *Biochem. Biophys. Acta* **2002**, *1575*, 135-138.

(10) Lovenberg, T. W.; Roland, B. L.; Wilson, S. J.; Jiang, X.; Pyati, J.; Huvar, A.; Jackson, M. R.; Erlander, M. G. Cloning and Functional Expression of the Human Histamine H_3 Receptor. *Mol. Pharmacol.* **1999**, *55*, 1101-1107.

(11) Cogé, F.; Guénin, S. P.; Rique, H.; Boutin, J. A.; Galizzi, J. P. Structure and Expression of the Human Histamine H_4-Receptor Gene. *Biochem. Biophys. Res. Commun.* **2001**, *284*, 301-309.

(12) O'Reilly, M.; Alpert, R.; Jenkinson, S.; Gladue, R. P.; Foo, S.; Trim, S.; Peter, B.; Trevethick, M.; Fidock, M. Identification of a Histamine H_4 Receptor on Human Eosinophils-Role in Eosinophils Chemotaxis. *J. Recept. Signal Transduction Res.* **2002**, *22*, 431-448.

(13) Hofstra, C. L.; Desai, P. J.; Thurmond, R. L.; Fung-Leung, W.-P. Histamine H_4 Receptor Mediates Chemotaxis and Calcium Mobilization of Mast Cells. *J. Pharmacol. Exp. Ther.* **2003**, *305*, 1212-1221.

(14) Gantner, F.; Sakai, K.; Tusche, M. W.; Cruikshank, W. W.; Center, D. M.; Bacon, K. B. Histamine H_4 and H_2 Receptors Control Histamine-Induced Interleukin-16 Release from Human $CD8^+$ T Cells. *J. Pharm. Exp. Ther.* **2002**, *303*, 300-307.

(15) Hashimoto, T.; Harusawa, S.; Araki, L.; Zuiderveld, O. P.; Smit, M. J.; Imazi, T.; Takashima, S.; Yamamoto, Y.; Sakamoto, Y.; Kurihara, T.; Leurs, R.; Bakker, R. A.; Yamatodani, A. A Selective Human H_4-Receptor Agonist: (-)-2-Cyano-1-methyl-3-{(2*R*,5*R*)-5-[1*H*-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine. *J. Med. Chem.* **2003**, *46*, 3162-3165.

(16) Jablonowski, J. A.; Grice, C. A.; Chai, W.; Dvorak, C. A.; Venable, J. D.; Kwok, A. K.; Ly, K. S.; Wei, J.; Baker, S. M.; Desai, P. J.; Jiang, W.; Wilson, S. J.; Thurmond, R. L.; Karlsson, L.; Edwards, J. P.; Lovenberg, T. W.; Carruthers, N. I. The First Potent and Selective Non-Imidazole Human Histamine H_4 Receptor Antagonists. *J. Med. Chem.* **2003**, *46*, 3957-3960.

(17) Thurmond, R. L.; Desai, P. J.; Dunford, P. J.; Fung-Leung, W.-P.; Hofstra, C. L.; Jiang, W.; Nguyen, S.; Riley, J. P.; Sun, S.; Williams, K. N.; Edwards, J. P.; Karlsson, L. A Potent and Selective Histamine H_4 Receptor Antagonist with Anti-Inflammatory Properties. *J. Pharm. Exp. Ther.* **2004**, *309*, 404-413.

(18) Terzioglu, N.; Van Rijn, R. M.; Bakker, R. A.; De Esch, I. J. P.; Leurs, R. Synthesis and Structure-activity Relationships of Indole and Benzimidazole Piperazines as Histamine H_4 Receptor Antagonists. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5251-5256.

(19) Black, J. W.; Duncan, W. A. M.; Durant, G. J.; Ganellin, C. R.; Parsons, M. E. Definition and Antagonism of Histamine H_2 Receptors. *Nature* **1972**, *236*, 385-390.

(20) Hill, S. J.; Ganellin, C. R.; Timmerman, H.; Schwartz, J. C.; Shankley, N. P.; Young, J. M.; Schunack, W.; Levi, R.; Haas, H. L. International Union of Pharmacology. XIII. Classification of Histamine Receptors. *Pharmacol. Rev.* **1997**, *49*, 253-278.

(21) Marquart, A. L.; Podlogar, B. L.; Huber, E. W.; Demeter, D. A.; Peet, N. P.; Weintraub, H. J. R.; Angelastro, M. R. Synthesis of Chiral Hydroxylated Quinozilidines via

Vinylogous Bischler-Napieralski Nitrilium Ion Cyclizations. *J. Org. Chem* **1994**, *59*, 2092-2100.

(22) Wolin, R.; Connolly, M.; Afonso, A.; Hey, J. A.; She, H.; Rivelli, M. A.; Williams, S. M.; West, R. E. J. Novel H_3 Receptor Antagonists. Sulfonamide Homologs of Histamine. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2157-2162.

(23) Kitbunnadaj, R.; Zuiderveld, O. P.; De Esch, I. J. P.; Vollinga, R. C.; Bakker, R. A.; Lutz, M.; Spek, A. L.; Cavoy, E.; Deltent, M.-F.; Menge, W. M. P. B.; Timmerman, H.; Leurs, R. Synthesis and Structure-Activity Relationships of Conformationally Constrained Histamine H_3 Receptor Agonists. *J. Med. Chem.* **2003**, *46*, 5445-5457.

(24) Govoni, M.; Lim, H. D.; El-Atmioui, D.; Menge, W. M. P. B.; Bakker, R. A.; Timmerman, H.; Leurs, R. A Chemical Switch for the Modulation of the Functional Activity of Higher Homologs of Histamine on the Human Histamine H_3 Receptor: Effect of Various Substitutions at the Primary Amino Function. **to be published**.

(25) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Anal. Biochem.* **1976**, *72*, 248-254.

(26) Van der Goot, H.; Schepers, M. J. P.; Sterk, G. J.; Timmerman, H. Isothiourea Analogues of Histamine as Potent Agonists or Antagonists of the Histamine H_3-Receptor. *Eur. J. Med. Chem.* **1992**, *27*, 511-517.

(27) Howson, W.; Parsons, M. E.; Raval, P.; Swayne, G. T. G. Two Novel, Potent and Selective Histamine H_3 Receptor Agonists. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 77-78.

(28) Van der Goot, H.; Timmerman, H. Selective Ligands as Tools to Study Histamine Receptors. *Eur. J. Med. Chem.* **2000**, *35*, 5-20.

(29) Durant, G. J.; Emmet, J. C.; Miles, P. D.; Parsons, M. E.; Prain, H. D.; White, G. R. Cyanoguanidine-Thiourea Equivalence in the Development of the Histamine H_2-Receptor Antagonists, Cimetidine. *J. Med. Chem.* **1977**, *20*, 901-906.

(30) King, R. J.; White, G. R. 2H-Imidazole-2-Thione Derivatives.; Smith Kline & French Laboratories Limited, Welwyn Garden City, England: United States, 1979.

(31) Uveges, A. J.; Kowal, D.; Zhang, Y.; Spangler, T. B.; Dunlop, J.; Semus, S.; Jones, P. G. The Role of Transmembrane Helix 5 in Agonist Binding to the Human H_3 Receptor. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 451-458.

(32) Nguyen, T.; Shapiro, D. A.; George, S. R.; Setola, V.; Lee, D. K.; Cheng, R.; Rauser, L.; Lee, S. P.; Lynch, K. R.; Roth, B. L.; O'Dowd, B. F. Discovery of a Novel Member of the Histamine Receptor Family. *Mol. Pharmacol.* **2001**, *59*, 427-433.

(33) Shin, N.; Coates, E.; Murgolo, N. J.; Morse, K. L.; Bayne, M.; Strader, C. D.; Monsma, F. J. J. Molecular Modeling and Site-Specific Mutagenesis of the Histamine-Binding Site of the Histamine H_4 Receptor. *Mol. Pharmacol.* **2002**, *62*, 38-47.

(34) Vollinga, R. C.; Menge, W. M. B. P.; Timmerman, H. A New Convenient Route for the Synthesis of 4(5)-(ω-Aminoalkyl)-1*H*-Imidazoles. *Recl. Trav. Chim. Pays-Bas* **1993**, *112*, 123-125.

Chapter 6

Synthesis of 4-substituted imidazoles on a solid support: optimisation of solid-phase reactions and generation of a small library

Marinella Govoni,[a] Edith Gelens,[a] Herman D. Lim,[a] Wiro M.P.B. Menge,[a] Remko A. Bakker,[a] Rob Leurs[a], Harry C.J. Ottenheijm[b] and Henk Timmerman[a]

[a] Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

[b] Scientific Development Group, NV Organon, PO Box 20, 5340 BH Oss, The Netherlands

Abstract

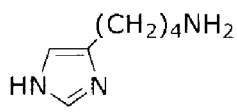
In view of the many predicted therapeutic applications of histamine H_3 and H_4 receptor (ant)agonists, the availability of novel ligands for those receptors showing a wide range of pharmacological activities is of high interest for the determination of the H_3 and H_4 receptor pharmacophore and therefore for the development of new, potent and selective drugs. Carbon-carbon bond formation is of particular importance for the synthesis of small organic molecules used in drug discovery research. Palladium catalyzed processes have often been used for such reactions and therefore appear to be a fruitful area for extension to solid-phase synthesis (SPS). We present here both a SPS route for the development of imbutamine and impentamine derivatives and the application of a palladium catalyzed cross coupling reaction on a solid support for the generation of a library of conformationally constrained analogues of histamine, bearing a triple bond attached directly to the imidazole ring in the 4-position. The compounds (**19a-v**) were obtained in moderate to good yields (37-62%) and moderate to excellent purities (37-95%). The novel ligands were pharmacologically investigated in SK-N-MC cells stably expressing the human H_3 or H_4 receptor and a cyclic AMP responsive element (CRE)-β-galactosidase reporter-gene. Although none of the compounds in our library exhibit a high affinity or potency for either of the two receptors, they do possess an interesting scaffold that can be easily synthesised and modified with a high variety of substituents via parallel polymer-supported techniques. Moreover, we report for the first time a solution-phase, palladium-catalyzed cross-coupling reaction between an unprotected 4-iodoimidazole and terminal aliphatic and aromatic alkynes.

Introduction

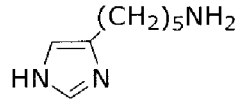
The different pharmacological actions of histamine are mediated via the activation of at least four distinct receptor subtypes, namely H_1, H_2, H_3[1-3] and H_4 receptors.[4] The histamine H_3 receptor (H_3R) controls neuronal synthesis of histamine and regulates its release into the synaptic cleft.[5] Furthermore, this receptor modulates the release of other neurotransmitters, such as acetylcholine,[6] dopamine,[7] serotonin[8] and noradrenaline,[9] in both central and peripheral nervous systems. Therefore, the H_3R is considered a potential target for several diseases and neurological disorders,[10] including epilepsy, schizophrenia,[11] eating and drinking disorders,[12] arousal and sleep disorders,[13] memory and learning deficits,[14,15] and Alzheimer's disease.[16] In 2000, a fourth histamine receptor subtype, the histamine H_4 receptor (H_4R), was identified, and shown to be mainly expressed on leukocytes.[4] The H_4R is suggested to be a new therapeutic target for the regulation of immune functions with possible uses in inflammation, allergy and asthma.[17]

In chapter 4 we have presented the synthesis and pharmacological characterization of several 4-substituted imidazoles behaving as H_3R and H_4R ligands and we demonstrated that very subtle changes at the substitution in the side chain nitrogen or in the length of the side chain alter can enormously the pharmacological activity of the ligands. This resulted in a series of compounds spanning the whole spectrum of activities, from full agonism to full inverse agonism, which underlined the usefulness of the compounds presented in that study as pharmacological tools. Moreover, considering the several possible therapeutic applications of H_3 and H_4 receptor (ant)agonists, the availability of a series of ligands with such a wide range of pharmacological activities is of high interest for the development of new, potent and selective H_3 and H_4 selective compounds.

In view of the relevance of such a series of ligands, and therefore the need that may arise for the synthesis of related compounds, we present here a solid-phase synthesis (SPS) route for the development of derivatives of both imbutamine and impentamine (Figure 1) derivatives.



Imbutamine



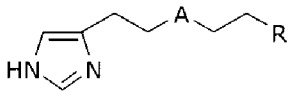
Impentamine

Figure 1. Structures of 4-[1*H*-imidazol-4-yl]-butylamine (imbutamine) and 5-[1*H*-imidazol-4-yl]-pentylamine (impentamine).

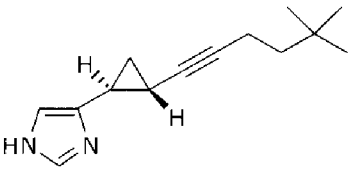
In recent years the SPS of small organic molecules has become very important because of the utility of this methodology for producing combinatorial libraries,[18] a strategy that has proved very successful for generating new leads and accelerating the process of drug discovery.[19,20] While the formation of amide bonds on a solid support has been optimized, other types of

reactions have not been studied as extensively. Carbon-carbon bond formation is of particular importance for the synthesis of small organic molecules; palladium catalyzed processes have been used for the above reaction class,[21-24] in particular for the coupling of imidazole to alkynyl and vinyl substrates,[25-27] and therefore appear to be a fruitful method for extension to SPS.

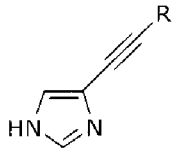
It has been demonstrated that potent and selective H_3R ligands possess distinct stereochemical characteristics.[28-30] The development of rigid histamine analogues will therefore likely contribute to the determination of the H_3R and H_4R pharmacophores. Due to its structural features, the cyclopropane ring has been used to improve H_3R affinity, and the activity of some cyclopropanic derivatives like the agonist cyclopropylhistamine[31] or the potent chiral alkyne derivative GT-2331 (cipralisant, Perceptin®)[32,33] has been reported (Figure 2). GT-2331 has reached Phase II clinical trials for the treatment of ADHD.[34]



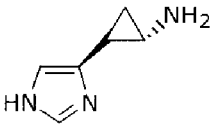
GT-2321: A = CH_2CH_2; R = *c*-hexyl
GT-2227: A = CH=CH; R = *c*-hexyl
GT-2327: A = CH=CH; R = phenyl
GT-2286: A = C≡C ; R = *c*-pentyl
GT-2293: A = C≡C ; R = *t*-butyl



GT-2331



alkynylimidazoles 19a-v



Cyclopropylhistamine

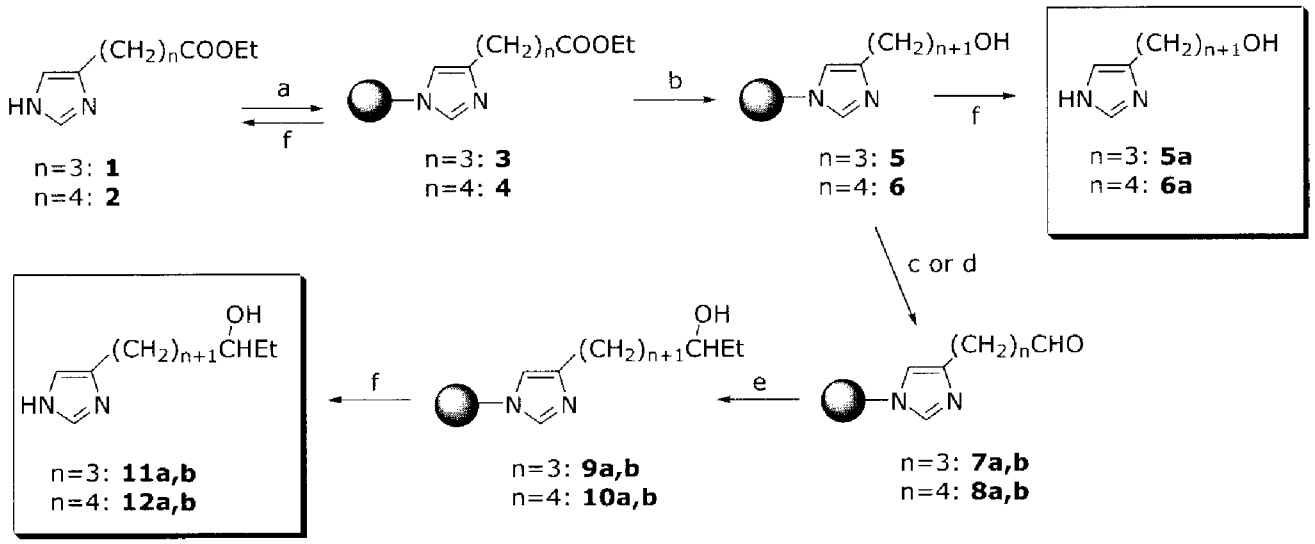
Figure 2. Structures of the 4-substitued imidazoles GT-2321, GT-2227, GT-2327, GT-2286, GT-2293, GT-2331[48] (cipralisant, Perceptin®), 2-(1*H*-imidazol-4-yl)-cyclopropyl amine (cyclopropylhistamine), and the general formula of the alkynylimidazoles **19a-v** (see also Table 2).

In this study we present the application of a palladium catalyzed cross coupling reaction on a solid support for the generation of a library of conformationally constrained analogues of histamine, bearing a triple bond directly connected to the 4-position of the imidazole ring. The synthesized compounds were evaluated against the full agonist histamine and the full inverse agonist thioperamide in SK-N-MC cells stably expressing either human H_3Rs or H_4Rs and a cyclic AMP responsive element (CRE)-β-galactosidase reporter-gene.

Approaches towards the solid-phase synthesis of imbutamine and impentamine derivatives

Chemistry

Secondary alcohols **11a,b** and **12 a,b**, used to study the conversions of the immobilized alcohols **5** and **6** into the relatively labile resin bound aldehydes **7** and **8**, were prepared according to the route shown in Scheme 1.

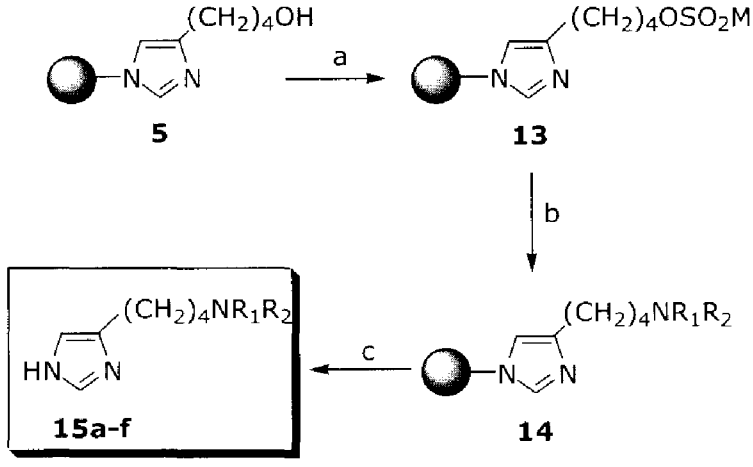


Scheme 1. Synthesis of compounds **11a,b** and **12a,b**. Reagents: (a) 2-Chlorotrityl resin, DIPEA, NMP, DCM; (b) $LiAlH_4$, THF, reflux; (c) Swern oxidation; (d) $Pyr.SO_3$, Et_3N, DCM; (e) EtMgBr, THF; (f) 20% TFA/DCM.

First, 4-(1*H*-imidazol-4-yl)-butyric acid ethyl ester **1**[35] or 5-(1*H*-imidazol-4-yl)-pentanoic acid ethyl ester **2**[35] was immobilized onto the commercially available 2-chlorotrityl resin, using di-isopropylethylamine (DIPEA) as base and DCM/N-methylpyrrolidinone (NMP) as solvent mixture. The degree of coupling and loading of the resin (necessary to calculate the amount of reagents needed in the steps hereafter) was determined by the weight gain of the resin after coupling and by cleavage of the resin bound esters to give back the starting esters **1** and **2**. The immobilized esters **3** and **4** were then reduced to the corresponding alcohols (**5** and **6**) by refluxing with 8 equivalents of lithium aluminumhydride ($LiAlH_4$) in THF. A small sample of resin bound alcohols **5** and **6** was taken to generate alcohols **5a** and **6a** by cleavage from the solid support, in order to check the completion of the reduction reaction. Next, alcohols **5** and **6** were converted to aldehydes **7** and **8** via two different kinds of oxidation reactions. Aldehydes **7a** and **8a** were obtained through a Swern oxidation, whereas oxidation with pyridine sulphur trioxide was used to obtain aldehydes **7b** and **8b**. Aldehydes **7** and **8** were

subsequently alkylated by treatment with 10 equivalents of ethyl magnesium bromide in THF resulting in compounds **9** and **10**. Cleavage from the support with 20% trifluoroacetic acid (TFA) in DCM gave the secondary alcohols **11** and **12** in 35 to 71% yields and 18 to 38% conversions.

The generation of a small (6 compounds) library of aminoalkylimidazoles (**15a-f**) is depicted in Scheme 2.



Scheme 2. Synthesis of compounds **15a-f**. Reagents: (a) $MeSO_2Cl$, pyridine, DCM; (b) NHR_1R_2, NMP, 60 °C; (c) 20% TFA/DCM.

The hydroxy group of immobilized alcohol **5** was readily converted into a suitable leaving group, such as the mesylate group, by treatment with 10 equivalents of methanesulfonyl chloride ($MeSO_2Cl$) in dry DCM and dry pyridine, yielding protected alcohol **13**. Heating of resin **13** with a suitable amine (50 equivalents) in dry NMP and subsequent cleavage of immobilized aminoalkylimidazoles **14** under acidic conditions (20% TFA/DCM) generated compounds **15a-f** in moderate yields (16 to 48%) and conversions (1 to 69%).

Results and discussion

Prior to this study, a SPS route towards imbutamine and impentamine derivatives was not available. However, imbutamine and impentamine derivatives have been synthesised via a solution-phase synthesis following the pathways described in chapter 4 (Schemes 1 and 2). For the solid-phase synthesis of imbutamine and impentamine derivatives we studied whether the described methods[35] could be transferred to solid-phase synthesis. The commercially available 2-chlorotrityl resin was our resin of choice, because of its similarity to the well-known trityl protective group used in solution, its high loading capacity and, since in the step before the removal of the protective group acetic acid should be used in equimolar amounts, its stability towards acids.

The first step of the solid-phase synthetic route, after anchoring esters **1** and **2** to the resin, was the reduction of the immobilized esters **3** and **4** to alcohols **5** and **6**, which proceeded smoothly using $LiAlH_4$ as reducing agent and dry THF as a solvent (Scheme 1). The success of the reaction was evaluated via estimation of the degree of conversion of the starting esters into the alcohols. This was achieved through a sample cleavage of alcohols **5** and **6**, under acidic condition (20% TFA) to give resin free compounds **5a** and **6a**, which pointed out a quantitative conversion.

Next, the oxidation of alcohols **5** and **6** to aldehydes **7** and **8** was investigated (Scheme 1). Two different kinds of oxidation reactions have been tried, the Swern oxidation and an oxidation with pyridine sulfur trioxide. To estimate the conversion from alcohols **5** and **6** to the relatively labile aldehydes **7** and **8**, the latter were alkylated with ethyl magnesium bromide directly after the oxidation step, giving secondary alcohols **9** and **10** (Scheme 1). This was necessary to prevent the aldehyde group from engaging in side reactions during TFA-cleavage or by oxidative exposure to air.

Swern oxidations have successfully been performed on the solid-phase.[36] For instance, Marx *et al.*[37] used a Swern oxidation in the synthesis of polycyclic lactams, in which an alcohol group was transformed to the corresponding aldehyde. In this case, the resin bound alcohol was added to the reactive intermediate of the Swern reaction (a dimethylchlorosulfonium anion) as a suspension in dichloromethane, which ensures an optimal swelling of the resin. However, this procedure turned out to be technically unfeasible in our reaction set-up, because the suspended resin is very sticky and it proved impossible to transport the complete batch to a single glass reaction vessel without losing resin beads in the syringe. We therefore decided to proceed switching the chain of events, and add a solution of the reactive intermediate to the alcohol anchored to the resin instead. At first, the dimethylchlorosulfonium anion, key intermediate of the Swern oxidation, was prepared using 8 equivalents of oxalylchloride and 16 equivalents of DMSO in dry DCM at -60 °C. The resulting solution was added dropwise to the swollen resin **5** or **6** at -60 °C. After 1 hour at -60 °C, triethylamine (20 equivalents) was added and the reaction mixture was stirred for an additional hour. The reaction was then allowed to warm to room temperature and left overnight. After washing, a small resin sample (**7a** or **8a**) was removed for IR-analysis. The IR-spectrum clearly showed a strong absoption at about 1720 cm^{-1}, for the aldehyde carbonyl. The remainder of the batch was then treated with 10 equivalents of ethyl magnesium bromide in dry THF, to give secondary resin bound alcohols **9a** and **10a**. Next, the resin was washed and treated with 20% TFA to cleave off the product, yielding compounds **11a** and **12a** (Scheme 1). Following evaporation of the solvents, the product mixture was analysed by ^{1}H-NMR and ESI-MS, which underlined a good yield (56-59%), but a moderate conversion (25-26%) of aldehydes **7a** and **8a**. In an effort to improve the conversion degree, this reaction step was modified by adding the dimethylchlorosulfoniun anion solution at once. We hypothesised that this could prevent the dimethylchlorosulfoniun

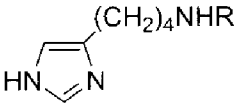
anion from a rapid degradation due to modified temperature conditions. Unfortunately, also in this case the reaction did not go to completion, though slightly better conversions (38-40%) and yields (68-71%) were observed. In order to achieve better conversions and yields we tried the use of a trityl Tentagel® instead of the polystyrene equivalent. This resin had not been our first choice because of its low stability towards acidic (cleavage) conditions and its low loading capacity of approximately 0.20 mmol/g. This low loading capacity is inherent to Tentagel® resins. However, Tentagel® resins do present an interesting advantage over their polystyrene analogues: the sterical hindrance from the polymeric backbone is highly reduced in Tentagel® resins. This prompted us to try this resin in our experimental set-up, following the reasoning that the reagents might be too bulky to reach all reaction sites in the pores of the resin. Yet, even this modification failed to give the desired increase of yields and conversions, which remained as low as 10%.

Still needing an efficient way to convert alcohols **5** and **6** into aldehydes **7** and **8**, we decided to change the kind of oxidation reaction used, exploring the feasibility of oxidation of immobilized alcohols with pyridine sulphur trioxide. Sulphur trioxide can oxidize alcohols to the corresponding aldehydes with a reaction mechanism similar to that of the Swern oxidation.[38] The Swern reaction only proceeds at low temperatures (-60 °C), whereas the oxidation with pyridine sulfurtrioxide gives excellent results at room temperature, which makes the reaction much easier to perform, especially on a solid support.[39] Resin **5** or **6** was swollen in dry DCM and 9 equivalents of triethylamine were added. Next, 9 equivalents of pyridine sulphurtrioxide in DMSO were added and the reaction was left at r.t. for 72 hours. After washing, a small sample of resin **7b** or **8b** was removed for IR-spectroscopy, whilst the rest of the resin was alkylated with ethyl magnesium bromide as described earlier yielding secondary alcohols **9b** and **10b**. The cleavage products **11b** and **12b** (Scheme 1) were analyzed by ^{1}H-NMR and ESI-MS. The conversion for both aldehydes was 25-30% and the yield 40-45%. Again, the IR-spectrum of the resin showed a clear aldehyde-peak centered around 1720 cm^{-1}. Changing the order of addition or the relative amounts of the reagents did not improve the conversions and yields of the reaction.

Since the preparation of the imidazole aldehydes **7** and **8** necessary to perform a successive reductive amination[40-42] failed, we decided to explore another reaction scheme in order to obtain a library of imbutamine and impentamine derivatives. Our following effort consisted in converting the hydroxy group into a suitable leaving group, anticipating that this can be substituted for an amine (Scheme 2). The mesylate group was the protective group of choice, since the reaction conditions for the introduction of this group are relatively mild and because it can be easily exchanged. Resin **5** was swollen in dry DCM and a solution of 10 equivalent of methanesulfonyl chloride in dry pyridine was added. The reaction mixture was left at r.t. overnight, then the resin was washed and treated, in a pilot reaction, with 50 equivalents of isobutylamine in dry NMP at 60 °C for 24 hours. After washing, the desired product **15a**

(Scheme 1 and Table 1) was obtained in 69 % conversion and 34% yield following cleavage in acidic medium (20% TFA) for 48 hours. Variation of the reaction conditions (higher temperature and/or larger excess of amine) did not improve the conversion. Also, efforts to reproduce the initial successful reaction sequence failed. Moreover, a lot of the starting amine used as reagent often contaminated the cleavage product, as confirmed by ^{1}H-NMR analysis. Efforts to remove the excess of amine before cleavage failed. Other amines (cyclopropylamine, cyclopentylamine, cycloheptylamine, aniline and benzylamine,) were coupled in the last reaction step, to check whether the reaction with isobutylamine was an outlier. The results of these experiments are shown in Table 1. Cyclopentylamine, cycloheptylamine and benzylamine were coupled in low to moderate yield, as can be seen with products **15 c** (24% conversion, 43% yield), **15d** (25% conversion, 35% yield), and **15f** (17% conversion, 48% yield). Cyclopropylamine and aniline did not couple at all, compounds **15b** and **15e** both showing a conversion lower than 1% (Table 1).

Table 1. Chemical structures and results of the library synthesis of compounds **15a-f.**



compd	R	Conversion[a] (%)	Yield[b] (%)
15a	*i*-Butyl	69	34
15b	Cyclopropyl	<1	33
15c	Cyclopentyl	24	43
15d	Cycloheptyl	25	35
15e	Phenyl	<1	16
15f	Benzyl	17	48

[a] Determined by ^{1}H-NMR analysis of the ratio's of the imidazole peaks of the cleaved alcohol **5** and the product **15**; [b] Determined by weighing the crude product as cleaved from the solid support.

The reason for the poor coupling with cyclopropylamine (compound **15b**) might be the low boiling point of this amine. The unsuccessful coupling with aniline (compound **15e**) might instead be due to the low reactivity of the aromatic amine compared to the more nucleophilic aliphatic amines.

Since the initially obtained positive results in the reaction with isobutylamine were not reproducible, we stopped our efforts to the SPS of imbutamine and impentamine analogues. For the synthesis of those derivatives parallel solution phase chemistry proved to be much easier and highly reproducibile and it has been applied instead, as already discussed in Chapter 4.

Conclusions

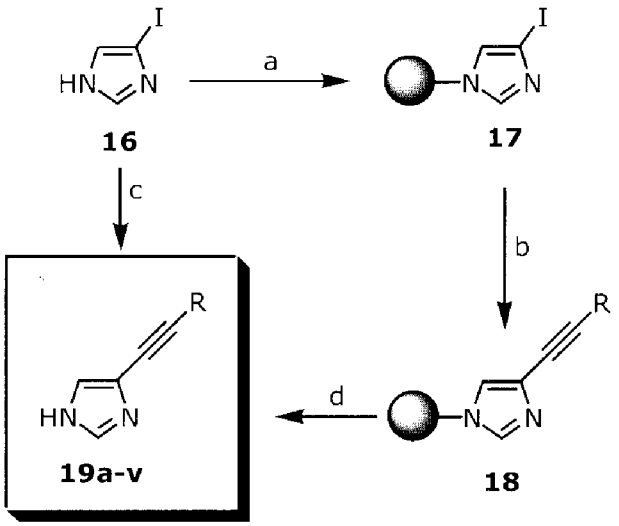
Efforts to develop a SPS route to imbutamine and impentamine (Figure 1) derivatives have been presented in this study. Trials of transforming resin bound alchohols **5** and **6** into aldehydes **7** and **8** via either a Swern or a pyridine sulphur trioxide oxidation (Scheme 1) ended in conversions of at best 40%. We considered that too a low value to proceed to the reductive amination step, which became therefore redundant.
In a different reaction pathway (Scheme 2), the exchange of the hydroxy group of alcohol **5** for the good leaving mesylate group and successive introduction of the desired amine was evaluated, in order to obtain library products **15a-f**. This new route did give in one case the desired product **15a** in moderate yield (34%) and very good conversion (69%). However, the coupling of the other amines to give aminoalkylimidazoled **15b-f** did not live up to the promises of the first pilot reaction with isobutylamine; conversions and yields were low, ranging respectively from less than 1% to 25% and from 16 to 48%, and the reaction steps were difficult to reproduce.
Due to the several problems we encountered and the fact that the solution phase chemistry of imbutamine and impentamine analogues proved to be much easier showing a higher reproducibility as well, the SPS approach was terminated. We therefore conclude that, despite our efforts, we have not been able to develop a suitable, easy and reproducible method for the solid-phase synthesis of impentamine derivatives **15** (Table 1).
The applicability of solid supported imidazoles synthesis towards medicinal chemistry depends greatly on the suitability of the synthesis route at hand. Many factors seem to play a role in the success of a SPS route, but the penetration of the reagents to the pores of the resin seems to be the most important. If a poor penetration of the reagents is observed, solid-phase synthesis is not a suitable technique and conventional solution-phase synthesis should be applied instead.

Parallel synthesis of conformationally constrained aliphatic and aromatic alkynyl-imidazoles via palladium-catalyzed cross-coupling reactions on a solid support.

Carbon-carbon bond formation is of particular importance for the synthesis of small organic molecules. Palladium catalyzed processes have been used for the above reaction class,[21-24] and appear to be a fruitful method for extension to SPS.
We present here the application of a palladium catalyzed cross coupling reaction on a solid support for the generation of a library of conformationally constrained analogues of histamine, bearing a triple bond next to the imidazole ring in the 4-position.

Chemistry

4-iodoimidazole **16**[23] was attached to 2-chlorotrityl resin with DIPEA in NMP/DCM at r.t. for 20 h to give resin **17**,[43] as shown in Scheme 3.



Scheme 3. Synthesis of compounds **19a-v**. Reagents: (a) 2-Chlorotrityl resin, DIPEA, NMP, DCM; (b) $Pd(PPh_3)_4$, alkyne, Et_3N, NMP, 70 °C; (c) $Pd(PPh_3)_4$, alkyne, CuI, Et_3N, NMP, 80 °C; (d) 20% TFA/DCM.

The degree of coupling and loading of the resin (necessary to calculate the amount of reagents needed in the steps hereafter) was determined by the weight gain of the resin after coupling and by cleavage of the resin bound starting reagent to give back 4-iodoimidazole **16**. The palladium catalysed reactions of compound **17** and 50 equivalents of the appropriate terminal alkyne in the presence of triethylamine (Et_3N, 50 equivalents) and tetrakis(triphenylphospine) palladium(0) ($Pd(PPh_3)_4$, 20 mol%) in degassed NMP gave the cross-coupled products **18**, which were successively cleaved off the solid support under acidic conditions (20% TFA) to give alkynylimidazoles **19a-v** in 37-95% conversion and 37-62% yield.
Final compounds **19a,b,m** were re-synthesised in a solution-phase setting according to the procedure of Sonogashira.[44] As shown in Scheme 3, heating of 4-iodoimidazole **16** at 80 °C for 3 h in the presence of $Pd(PPh_3)_4$ (10 mol%), copper iodide (CuI, 10 mol%) and degassed Et_3N (10 equivalents) in degassed NMP gave products **19a,b,m** in moderate yields (14-35%).

Results and discussion

Recent studies identified the 4-substituted imidazoles depicted in Figure 2 as compounds showing good affinity for the H_3R and acting as antagonists at the rat H_3R.[45-47] Especially the ligands bearing a triple bond between the C3 and C4 carbon atoms of the side chain (A) were

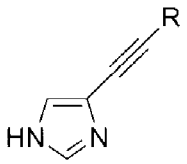
found to have a higher H_3R affinity, compared to those with a double bond or a saturated moiety. Introduction of a cyclopropane ring between the C1 and C2 of the side chain of GT-2293 (Figure 2) turned this semi-rigid histamine analogue into the conformationally constrained compound GT-2331 (Figure 2).[32,33,48] This chiral alkyne derivative behaves as a potent antagonist on the rat H_3R.[49] However, Fox and coworkers have recently reported on the partial H_3R agonistic behavior of GT-2331.[50] Perceptin® has already entered Phase II clinical trials for the treatment of ADHD.[34]

These developments open up many new possibilities for the synthesis of novel acetylene-based imidazoles. Several changes can be made to the aforementioned acetylene-based ligands to potentially obtain novel compounds. By shifting the triple bond of those ligands from the C3-C4 to the C1-C2 position in the side chain, a series of 4-alkynylimidazoles (**19a-v**) can e.g. be obtained (Figure 2).

Previous methods for preparing 4-substituted imidazoles involved the use of transmetallation reactions of protected 4-haloimidazoles, using either lithium[51] or Grignard reagents.[52,53] Neither of those methods were practical for our purposes however, as we required the introduction of unsaturated side chains. Palladium catalysed coupling reactions[25] of 2-chlorotrityl resin bound 4-iodoimidazole **17**[43] (Scheme 3) with aliphatic and aromatic terminal alkynes were therefore examined and the results of this investigation are presented in Table 2. Resin bound compound **18** was washed as usual after cross-coupling reactions, but additional wash steps with toluene in DCM and 0.5% TFA in DCM were applied to remove all traces of the palladium catalyst. The coupling of the aryl- and alkenyl-substituted alkynes leading to ligands **19e-v** proceeded smoothly and conversions of over 95% and yields between 37 and 62% were obtained. The coupling of aliphatic alkynes, generating compounds **19a-d**, however, was less successful: the yields were 44-55% and the conversions were somewhat lower, ranging from 37 to 55%. We observed similar results in a previous study on Heck reactions with aliphatic alkynes (unpublished results). One might reason that the low boiling points of these alkynes caused their removal from the reaction mixture by evaporation. However, this hypothesis is likely not true as the addition of more alkyne or lowering the reaction temperature had no positive effect on the conversions. It has been reported that aliphatic alkynes, which are more reactive than the aromatic analogues, can react with each other in the presence of a palladium catalyst.[54] Cliff and coworkers reported that the palladium(0)-catalysed coupling of 1-(ethoxymethyl)-4-iodoimidazole with aliphatic or alicyclic alkynes in the presence of copper(I) iodide and triethylamine in acetonitrile at reflux gave the desired cross coupled 4-alkynyl-substituted imidazole in 23-40% yield, but that also the bialkyne was formed from the homocoupling of the starting aliphatic alkyne used as reactant in 35-69% yield.[26,55] Furthermore, reactions in solution generally proceed faster than reactions on the solid support. The competition between the coupling of the alkyne with the

4-iodoimidazole and the homocoupling of two molecules of alkyne might indeed explain the lower conversions observed for imidazoles **19a-d** (Table 2).

Table 2. Chemical structures and results of the library synthesis of compounds **15a-f.**



compd	R	Conversion[a] (%)	Yield (%)
19a	Propyl	37	44
19b	Butyl	45	55
19c	Pentyl	55	50
19d	Methoxymethyl	50	50
19e	Benzyl	43	39
19f	Cyclopent-1-enyl	>95	45
19g	Cyclohex-1-enyl	>95	53
19h	Phenyl	>95	50
19i	4-Tolyl	>95	62
19j	3-Methoxyphenyl	>95	54
19k	4-Methoxyphenyl	>95	55
19l	3-Trifluoromethylphenyl	>95	48
19m	4-Trifluoromethylphenyl	>95	45
19n	2-Fluorophenyl	>95	45
19o	4-Fluorophenyl	>95	48
19p	2,4-Difluorophenyl	>95	41
19q	2-Chlorophenyl	>95	42
19r	4-Chlorophenyl	>95	54
19s	3-Aminophenyl	83	37
19t	4-Aminophenyl	>95	37
19u	1-Naphtyl	>95	58
19v	3-Pyridyl	>95	45

[a] Determined by ^{1}H NMR, remainder is 4-iodomidazole or imidazole unless otherwise stated. [b] Determined by weighing the crude product as cleaved from the solid support.

In view of their potential therapeutic applications, ligands displayed in Table 2 have also been tested for their pharmacological activity on the human H_3R and H_4R. Preliminary screening of the crude compound mixtures obtained immediately after cleavage from the solid support was carried out on SK-N-MC cells stably expressing either the human H_3R[3] or the human H_4R[17] and a cyclic AMP responsive element (CRE)-β-galactosidase reporter-gene. Modulation of forskolin (1 μM)-stimulated cAMP production was established in the presence of 1, 10 and 100 μM of the compound of interest, as well as in the presence of 10 μM of the compound of interest plus the natural agonist histamine or the full inverse agonist thioperamide. Results obtained for the activity of the crude compounds on the human H_3 and H_4 receptors are shown in Figure 3; as can be seen from the graphs, most ligands were found to be (almost) inactive, but three of them, namely **19a,b,m**, showed an interesting level of H_3R activity (Figure 3A).

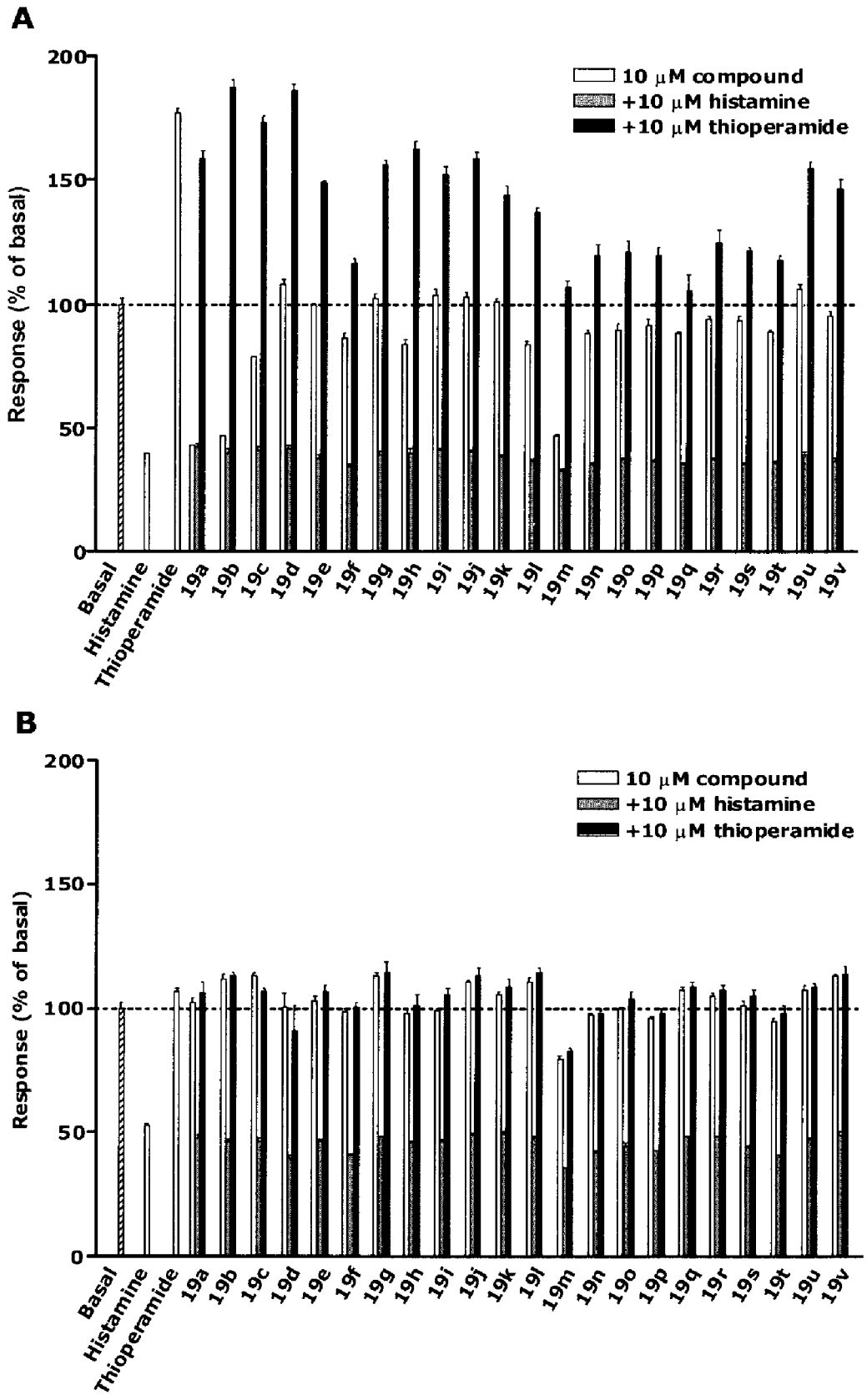


Figure 3. The effects of crude compounds **19a-v** on the basal signalling of the H_3R (A) and H_4R (B), as measured by CRE-mediated β-galactosidase reporter gene assay. Modulation of forskolin (1 μM)-stimulated cAMP production was evaluated either in the presence of 10 μM of the compound of interest, or in the presence of 10 μM of the compound of interest plus the natural agonist histamine (10 μM) or the full inverse agonist thioperamide (10 μM).

Thus, the crude mixture of the above mentioned compounds was tested on the human H_3R in a full dose-response curve in order to obtain an indication about the functional activity of the compounds (Table 3). All three compounds (**19a,b,m**) were found to show an agonistic behavior on the human H_3R (Figure 4).

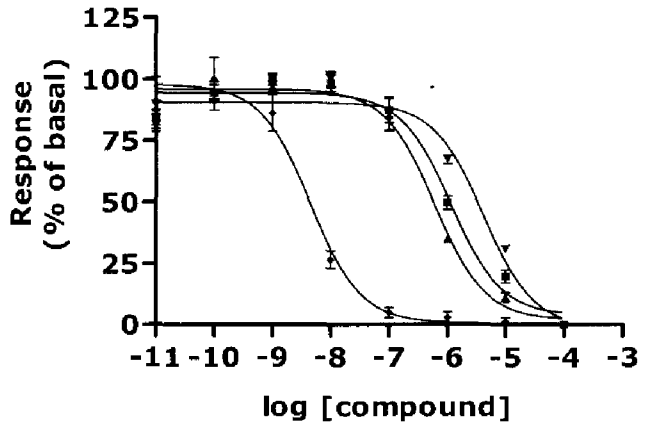


Figure 4. The effects of compounds **19a,b,m** on the basal signalling of the H_3R, as measured by CRE-mediated β-galactosidase reporter gene assay. Dose-response curves of the full H_3R agonists VUF 5908 (▲, **19a**), VUF 5909 (■, **19b**) and VUF 5924 (▼, **19m**) (see also Table 2 and 3). Representative dose-response curve of the full H_3R agonist histamine (♦) is shown. Data are normalised to the basal signalling observed in the assay (set to 100%).

It was therefore decided to re-synthesize these three derivatives via a parallel solution-phase approach in a convenient one-pot reaction according to the procedure of Sonogashira[44] (Scheme 3), in order to obtain the desired compounds with a higher degree of purity.

Many examples of solution-phase palladium-catalyzed cross-coupling reactions between 4-iodoimidazole and alkynes have been reported.[22,25-27,55-57] However, in all these cases the N1 position of the imidazole ring had to be protected by a variety of functional groups, such as trityl, benzesulfonyl, ethoxymethyl and *p*-tosyl, which were claimed to serve the double purpose of both activating the imidazole ring for carbon-carbon bond formation and protecting the reaction product in the subsequent reaction steps.[57] We here report for the first time a palladium-catalyzed cross-coupling reaction between an unprotected 4-iodoimidazole and terminal aliphatic and aromatic alkynes. The reaction proceeded smoothly, was extremely simple to perform, and the crude reaction mixture could be easily purified via flash chromatography, affording the target compounds in 36-49% yield. Notably, as the two time-consuming extra-steps (protection of 4-iodoimidazole and deprotection of the target compound) were avoided, the whole reaction pathway could be reduced to a one-pot reaction, leading to a remarkable time-saving procedure.

The three re-synthesised compounds were tested for their affinity on the human H_3 and H_4 receptors. As can be seen in Table 3 all the compounds showed a higher affinity for the H_4R, with a selectivity for the latter over H_3R ranging from 4- (**19m**) to 20-fold (**19b**).

Table 3. Pharmacological properties of compounds **19a,b,m** for the human H_3 (hH_3R) and H_4 (hH_4R) receptors. The values are expressed as means ± SEM of three separate experiments, each performed in triplicate, unless indicated otherwise.

	hH_3R			hH_4R		
compd (code)	pK_i	pEC_{50} [a,b]	i.a.[c]	pK_i	pEC_{50} [a,d]	i.a.[c]
19a (VUF5908)	3.8 ± 0.6	6.3 ± 0.1	agonist	4.7 ± 0.3	-[e]	-[e]
19b (VUF5909)	3.3 ± 0.9	5.8 ± 0.2	agonist	4.6 ± 0.6	-[e]	-[e]
19m (VUF5924)	4.2 ± 0.2	5.1 ± 0.3	agonist	4.8 ± 0.3	-[e]	-[e]

[a] Determined on the impure mixture of compound (see Table 2). [b] N=2. [c] Intrinsic activity. [d] N=1. [e] Not measurable.

However, in general, affinities of ligands **19a,b,m** for both receptors were relatively low, especially when compared to the acetylene-based ligands described earlier. Due to these poor affinities and to the low potency showed by these three compounds on the H_3R, as evaluated on the crude mixture obtained immediately after SPS (Table 3), it was decided not to proceed with further pharmacological characterization of the ligands. Nonetheless, compounds **19a,b,m** do possess an interesting new scaffold which is easily synthesized and can easily be varied with a high diversity of substituents.

Conclusions

The application of a palladium-catalyzed cross-coupling reaction on a solid support for the generation of a library of conformationally constrained analogues of histamine, bearing a triple bond attached directly in the 4-position of the imidazole ring, has been presented in this study. The compounds were obtained in moderate to good yields (37-62%) and moderate to excellent purities (37-95%). In view of their potential therapeutic applications, the novel ligands were pharmacologically investigated in vitro for their biological properties on the human H_3R and H_4R subtypes. Affinities were found to be relatively low for both receptors, but the synthesised compounds are still of interest, since they are slightly better for the H_4R than for the H_3R. These alkynylimidazoles could therefore be seen as lead compounds for a novel class of selective H_4R ligands. Moreover, although the synthesised ligands were not as active as hoped, they do possess an interesting scaffold that can be easily synthesised and modified with a high diversity of substituent via parallel polymer-supported techniques.

Finally, we here reported for the first time a solution-phase palladium-catalyzed cross-coupling reaction between an unprotected 4-iodoimidazole and terminal aliphatic and aromatic alkynes. The synthesis of the target compounds could be achieved via a one-pot procedure, which turned out to be consistently less time-consuming than the routes reported so far in literature to obtain alkynylimidazoles.

Experimental section

Chemistry

General procedures. Materials were obtained from commercial suppliers and used without further purification, unless otherwise stated. Solvents used were either AR or HPLC grade. Dry THF and dichloromethane (DCM) were freshly distilled under N_2 from $LiAlH_4$ and CaH_2, respectively. 2-Chlorotrityl chloride polystyrene resin was purchased from Novabiochem®.
Melting points were measured on an Electrothermal IA 9200 apparatus.
^1H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. Chemical shifts are given in ppm using the residual undeuterated solvent as reference.
Mass spectra were determined on a Perkin Elmer series 200 auto sampler.
Flash chromatography was performed on J.T.Baker Kieselgel 60.
Elemental analyses were performed by the Mikroanalytisches Labor Pascher, Remagen-Bandorf, Germany.
Solid-phase reactions were performed in MultiSynTech glass reaction vessels (5 mL) which were placed in fitting outer vessels (see Appendix A). The coupling of starting materials to the resins was performed in large glass reaction vessels (20 mL) that were placed in fitting outer vessels (see Appendix A).
All reactions were performed under an atmosphere of dry nitrogen.

Synthesis of imbutamine and impentamine derivatives

Compound 3

i) To a suspension of 2-chlorotrityl chloride resin (0.99 g, 1.09 mmol/g, 1.08 mmol) in dry DCM (8 mL) was added thionylchloride (1.5 mL). After 20 h the resin was filtered and washed with dry DCM (5x). ii) To a suspension of the above resin in dry DCM (10 mL) was added dropwise a solution of 4-(1*H*-Imidazol-4-yl)-butyric acid ethyl ester **1**[35] (1.04 g, 5.71 mmol, 5.3 equiv.) in NMP (3 mL) and diisopropylethylamine (3 mL). The mixture was bubbled under N_2 at room temperature (r.t.) for 20 h. The resin was subsequently filtered and washed with NMP (3x), 10% MeOH/DCM (3x), MeOH (3x), DCM (3x), MeOH (3x), DCM (3x), MeOH (2x), DCM (3x), MeOH (1x), DCM (3x), dry THF (3x), and diethylether (3x), and dried at 70 °C under vacuum to give resin **3**. The loading capacity of the resin after coupling was found to be 1.16 mmol/g. This was determined by cleavage from the support (20% TFA) to give product **1** as a trifluoroacetic acid salt. ^1H NMR ($CDCl_3/CD_3OD$): δ 1.20 (t, 3H, CH_3CH_2OCO), 1.45 (m, 2H, central CH_2), 1.62 (t, 2H, CH_2COO), 2.60 (t, 2H, imidazole-CH_2), 3.50 (q, 2H, CH_3CH_2OCO), 6.90 (s, 1H, imidazole-5H), 8.38 (s, 1H, imidazole-2H). LRMS: MS (ES^+) m/z =183.7 $(M + 1)^+$.

Compound 4

The same procedure as for compound **3** was employed with 0.99 g (1.09 mmol/g, 1.08 mmol) of 2-chlorotrityl chloride resin and 1.11 g (5.66 mmol, 5.2 equiv.) of 5-(1*H*-Imidazol-4-yl)-pentanoic acid ethyl ester **2**[35] to give resin **4**. The loading capacity of the resin after coupling was found to be 1.34 mmol/g, as determined by cleavage from the support (20% TFA) to give product **2** as a trifluoroacetic acid salt. ^{1}H NMR ($CDCl_3/CD_3OD$): δ 1.12 (t, 3H, CH_3CH_2OCO), 1.58 (m, 4H, central CH_2's), 2.22 (t, 2H, CH_2COO), 2.60 (t, 2H, imidazole-CH_2), 4.00 (q, 2H, CH_3CH_2OCO), 6.91 (s, 1H, imidazole-5H), 8.45 (s, 1H, imidazole-2H). LRMS: MS (ES^+) *m/z* =197.5 $(M + 1)^+$.

Compound 5

To a suspension of resin **3** (1.14 g, 1.32 mmol) in dry THF (10 mL) was added $LiAlH_4$ 1 M in THF (8 mL, 8 mmol, 6.1 equiv.). The reaction was heated under reflux for 8 hours, then left overnight at r.t. Following this the resin was filtered, washed with MeOH and 1 M HCl (3 x), and sonicated in an ultra sound bath. The resin was then washed with MeOH (3x), 10% MeOH/DCM (3x), MeOH (3x), DCM (3x), MeOH (3x), DCM (3x), MeOH (2x), DCM (3x), MeOH (1x), DCM (5x), and dried at 70 °C under vacuum to give resin **5**. To test whether the reaction was complete, some of the product was cleaved from the support (20% TFA) to give 4-(1*H*-imidazol-4-yl)-butanol trifluoroacetate **5a**. ^{1}H-NMR ($CDCl_3/CD_3OD$): δ 1.60 (m, 4H, central CH_2's), 2.60 (t, 2H, imidazole-CH_2), 4.18 (t, 2H, CH_2OH), 6.90 (s, 1H, imidazole-5H), 8.40 (s, 1H, imidazole-2H). LRMS: MS (ES^+) *m/z* =141.4 $(M + 1)^+$.

Compound 6

The same procedure as for compound **5** was employed using resin **4** (1.22 g, 1.63 mmol) and $LiAlH_4$ 1 M in THF (8 mL, 8 mmol, 4.9 equiv.) to give resin **6**. To test whether the reaction was complete, some of the product was cleaved from the support (20% TFA) to give 5-(1*H*-imidazol-4-yl)-pentanol trifluoroacetate **6a**. ^{1}H-NMR ($CDCl_3/CD_3OD$): δ 1.28-1.80 (m, 6H, central CH_2's), 2.60 (t, 2H, imidazole-CH_2), 4.25 (t, 2H, CH_2OH), 6.90 (s, 1H, imidazole-5H), 8.40 (s, 1H, imidazole-2H). LRMS: MS (ES^+) *m/z* =155.6 $(M + 1)^+$.

Compound 7

Swern oxidation (7a). i) A solution of oxalylchloride (1.18 mL) in dry DCM (10 mL) was cooled to –60 °C and added dropwise with a solution of DMSO (1.76 mL) in dry DCM (10 mL), then stirred for 10 minutes. ii) To a suspension of resin **5** (70 mg, 0.081 mmol) in dry DCM (2 mL) at –60 °C was added at once 2 mL of the above solution (corresponding to 8 equiv. of oxalylchloride and 16 equiv. of DMSO). The cold (-60 °C) mixture was stirred gently for 1 h, then added with triethylamine (0.23 mL, 1.66 mmol, 20 equiv.). After 1 h at –60 °C the mixture was allowed to warm to r.t. and left at r.t. overnight. The resin was filtered, washed

with MeOH (3x), H_2O (2x), MeOH (3x), 10% MeOH/DCM (3x), MeOH (3x), DCM (3x), MeOH (2x), DCM (3x), MeOH (1x), diethylether (6x), and dried under vacuum to give resin **7a**.

Oxidation with pyridine sulphurtrioxide (7b). To a suspension of resin **5** (90 mg, 0.10 mmol) in dry DCM (3.5 mL) were subsequently added triethylamine (0.28 mL, 2.02 mmol, 20 equiv.) and a solution of pyridine sulphurtrioxide (0.16 g, 1.01 mmol, 10 equiv.) in DMSO (1 mL), then the reaction mixture was gently stirred at r.t. After 48 h, the resin was filtered, washed with MeOH (3x), 10% MeOH/DCM (3x), MeOH (3x), DCM (3x), MeOH (2x), DCM (3x), MeOH (1x), DCM (3x), dry THF (3x), diethylether (3x) and dried under vacuum to give resin **7b**.

Compound 8

Swern oxidation (8a). The same procedure as for compound **7a** was employed using resin **6** (70 mg, 0.094 mmol) to give resin **8a**.

Oxidation with pyridine sulphurtrioxide (8b). The same procedure as for compound **7b** was employed using resin **6** (90 mg, 0.12 mmol) to give resin **8b**.

Compound 9a

To a suspension of resin **7a** (70 mg, 0.081 mmol) in dry THF (4 mL) was added ethyl magnesium bromide 3M in THF (0.27 mL, 0.81 mmol, 10 equiv.) and gently stirred at r.t. After 20 h the reaction mixture was sonicated in an ultra sound bath, then filtered. The resin was washed with saturated ammonium chloride solution, MeOH (3x), 10% MeOH/DCM (3x), DCM (3x), MeOH (2x), DCM (3x), MeOH (1x), DCM (3x), diethylether (3x) and dried at 70 °C under vacuum to give resin **9a**.

Compound 9b and 10a,b

The same procedure as for compound **9a** was employed using resin **7b** (90 mg, 0.10 mmol), **8a** (70 mg, 0.094 mmol) or **8b** (90 mg, 0.12 mmol) to give resins **9b** and **10a,b**.

Compounds 11 and 12, Cleavage from the Support.

Resins **9** and **10** were stirred in 20% TFA in DCM (4 mL) for 48 hours and then filtered. After removal of solvents the residues were dried at 70 °C under vacuum giving the crude products **11** and **12** in 35-71% yield.

6-(1*H*-Imidazol-4-yl)-hexan-3-ol trifluoroacetate (11a,b)

11a. Yield 65%. Conversion 21% (based on integrals in the ^{1}H-NMR). LRMS: MS (ES^+) *m/z* =169.6 $(M + 1)^+$.

11b. Yield 35%. Conversion 18% (based on integrals in the ^{1}H-NMR). LRMS: MS (ES^+) *m/z* =169.6 $(M + 1)^+$.

7-(1*H*-Imidazol-4-yl)-heptan-3-ol trifluoroacetate (12a,b)

12a. Yield 71%. Conversion 38% (based on integrals in the ^{1}H-NMR).

12b. Yield 44%. Conversion 29% (based on integrals in the ^{1}H-NMR).

Compound 13

To a suspension of resin **5** (80 mg, 0.093 mmol) in dry DCM (3.5 mL) were successively added 0.12 mL (1.48 mmol, 15 equiv.) dry pyridine and 75 μL (0.97 mmol, 10 equiv.) $MeSO_2Cl$. The mixture was gently stirred overnight at r.t. and then filtered. The resin was washed with MeOH (3x), 10% MeOH/DCM (3x), MeOH (3x), DCM (3x), MeOH (2x), DCM (3x), MeOH (1x), DCM (3x), diethylether (3x) and dried at 70 °C under vacuum to give resin **13**.

Compound 14

A suspension of resin **13** (80 mg, 0.0.093 mmol) in dry NMP (3.5 mL) was added with the primary amine (4.65 mmol, 50 equiv.) and heated at 60 °C. After 24 h the resin was filtered, washed with NMP (3x), MeOH (3x), THF (3x), MeOH (4x), DCM (2x), 10% toluene/DCM (4x), DCM (2x), MeOH (4x), 10% MeOH/DCM (4x), MeOH (4x), DCM (4x), MeOH (3x), DCM (4x), MeOH (2x), DCM (4x), MeOH (1x), DCM (5x), and dried at 70 °C under vacuum to give resin **14**.

Compound 15, Cleavage from the Support.

Resin **14** (60 mg, 0.070 mmol) was stirred in 20% TFA in DCM (4 mL) and a droplet of water for 48 hours and then filtered. After removal of solvents the residue was dried at 70 °C under vacuum giving the crude product 15 in 16-48% yield. Table 1.

[4-(1*H*-imidazol-4-yl)-butyl]-isobutyl-amine ditrifluoacetate (15a)

Yield 34%. Conversion 69% (based on integrals of the CH_2OH peak (starting material) and the $CH_2NR_1R_2$ peak (product) in the ^{1}H-NMR). LRMS: MS (ES^+) m/z =196.7 $(M + 1)^+$.

Cyclopropyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine ditrifluoacetate (15b)

Yield 33%. Conversion <1% (based on integrals of the CH_2OH peak (starting material) and the $CH_2NR_1R_2$ peak (product) in the ^{1}H-NMR).

Cyclopentyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine ditrifluoacetate (15c)

Yield 43%. Conversion 24% (based on integrals of the CH_2OH peak (starting material) and the $CH_2NR_1R_2$ peak (product) in the ^{1}H-NMR).

Cycloheptyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine ditrifluoacetate (15d)

Yield 35%. Conversion 25% (based on integrals of the CH_2OH peak (starting material) and the $CH_2NR_1R_2$ peak (product) in the ^{1}H-NMR).

Phenyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine ditrifluoacetate (15e)

Yield 16%. Conversion <1% (based on integrals of the CH_2OH peak (starting material) and the $CH_2NR_1R_2$ peak (product) in the ^{1}H-NMR).

Benzyl-[4-(1*H*-imidazol-4-yl)-butyl]-amine ditrifluoacetate (15f)

Yield 48 %. Conversion 17% (based on integrals of the CH_2OH peak (starting material) and the $CH_2NR_1R_2$ peak (product) in the ^{1}H-NMR).

Synthesis of acetylene-based imidazoles

Solid-Phase Synthesis

Compound 17

i) To a suspension of 2-chlorotrityl chloride resin (1.18 g, 1.09 mmol/g, 1.29 mmol) in dry DCM (8 mL) was added thionylchloride (1.5 mL). After 20 h the resin was filtered and washed with dry DCM (5x). ii) To a suspension of the above resin in dry DCM (5 mL) was added dropwise a solution of 4-iodoimidazole **16**[23] (1 g, 5.16 mmol, 4 equiv.) in NMP (3 mL), diisopropylethylamine (2 mL) and DCM (4 mL). The mixture was gently stirred at r.t. for 20 h. The resin was subsequently filtered and washed with NMP (3x), MeOH (4x), DCM (4x), MeOH (3x), DCM (4x), MeOH (2x), DCM (4x), MeOH, DCM (5x), diethylether (5x), then dried at 70 °C under vacuum to give resin **17**. The loading capacity of the resin after coupling was found to be 1.17 mmol/g. This was determined by cleavage from the support (20% TFA) to give product **16** as a trifluoroacetic acid salt. ^{1}H NMR ($CDCl_3/CD_3OD$): δ 7.20 (s, 1H, imidazole-5H), 8.22 (s, 1H, imidazole-2H).

Compound 18

To a suspension of resin **17** (80-90mg, 0.094-0.11 mmol) and tetrakis(triphenylphosphine)palladium (21.72-25.42 mg, 20 mol%) in degassed NMP (3 mL) were successively added the alkyne (4.7 mmol-5.5 mmol, 50 equiv.) and triethylamine (0.65 mL-0.76 mL, 4.7 mmol-5.5 mmol, 50 equiv.). The reaction mixture was heated to 70 °C and gently stirred for 18 h, then filtered. The resin was washed with NMP (3x), MeOH (3x), THF (3x), MeOH (4x), DCM (2x), 10% toluene/DCM (4x), DCM (2x), MeOH (4x), 10% MeOH/DCM (4x), MeOH (4x), DCM (2x), 0.5% TFA/DCM (4x), DCM (2x), MeOH (4x), 10% MeOH/DCM

(4x), MeOH (4x), DCM (4x), MeOH (3x), DCM (4x), MeOH (2x), DCM (4x), MeOH, DCM (5x), diethylether (5x), dry DCM (3x) and dried under vacuum at 70 °C to give resin **18**.

Compound 19, Cleavage from the Support.

Resin **18** was stirred in 20% TFA in DCM (4 mL) for 24 hours and then filtered. After removal of solvents the residue was dried at 70 °C under vacuum giving the crude product **19** as a TFA salt in 37-62% yield. Table 2. In some cases the TFA salt was transformed into a free base according to the following procedure. The crude product **19** was dissolved in water (3 mL), acidified with HCl (37%) and extracted with DCM (3 x 3 mL). The water layer was then basified with Na_2CO_3, saturated with NaCl and extracted with ethyl acetate (5 x 3 mL). The combined organic layers were dried ($MgSO_4$) and evaporated to dryness to give product **19** as free base.

4-Pent-1-ynyl-1*H*-imidazole trifluoroacetate (19a)

Yield 44%. Conversion 37%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.45 (s, 1H, imidazole-2H), 7.21 (s, 1H, imidazole-5H), 2.35 (t, 2H, $CCCH_2$), 1.55 (m, 2H, CH_2CH_3), 0.95 (t, 3H, CH_3). LRMS: MS (ES^+) *m/z* =135.8 $(M + 1)^+$.

4-Hex-1-ynyl-1*H*-imidazole (19b)

Yield 55%. Conversion 45%. ^{1}H-NMR ($CDCl_3$): δ 7.50 (s, 1H, imidazole-2H), 6.97 (s, 1H, imidazole-5H), 2.28 (t, 2H, $CCCH_2$), 1.50-1.27 (m, 4H, $CH_2CH_2CH_3$), 0.80 (t, 3H, CH_3). LRMS: MS (ES^+) *m/z* =149.7 $(M + 1)^+$.

4-Hept-1-ynyl-1*H*-imidazole (19c)

Yield 50%. Conversion 55%. ^{1}H-NMR ($CDCl_3$): δ 7.51 (s, 1H, imidazole-2H), 7.05 (s, 1H, imidazole-5H), 2.20 (t, 2H, $CCCH_2$), 1.60-1.15 (m, 6H, $CH_2CH_2CH_2CH_3$), 0.88 (t, 3H, CH_3). LRMS: MS (ES^+) *m/z* =163.8 $(M + 1)^+$.

4-(3-Methoxy-prop-1-ynyl)-1*H*-imidazole trifluoroacetate (19d)

Yield 50%. Conversion 50%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.31 (s, 1H, imidazole-2H), 7.32 (s, 1H, imidazole-5H), 4.20 (s, 2H, CH_2O), 3.32 (s, 3H, CH_3O). LRMS: MS (ES^+) *m/z* =169.7 $(M + 1)^+$.

4-(3-Phenyl-prop-1-ynyl)-1*H*-imidazole trifluoroacetate (19e)

Yield 39%. Conversion 43%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.35 (s, 1H, imidazole-2H), 7.30-7.00 (m, 6H, imidazole-5H + Ph-H), 3.78 (s, 2H, CH_2Ph). LRMS: MS (ES^+) *m/z* =182.2 $(M + 1)^+$.

4-Cyclopent-1-enylethynyl-1*H*-imidazole trifluoroacetate (19f)

Yield 45%. Conversion 95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.15 (s, 1H, imidazole-2H), 7.22 (s, 1H, imidazole-5H), 6.15 (m, 1H, cyclopent-2H), 2.50-2.30 (m, 4H, cyclopent-3H + -5H), 1.85 (q, 2H, cyclopent-4H). LRMS: MS (ES^+) *m/z* =159.8 $(M + 1)^+$.

4-Cyclohex-1-enylethynyl-1*H*-imidazole trifluoroacetate (19g)

Yield 53%. Conversion 95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.11 (s, 1H, imidazole-2H), 7.32 (s, 1H, imidazole-5H), 6.15 (m, 1H, cyclohex-2H), 2.12-1.95 (m, 4H, cyclohex-3H + -6H), 1.67-1.45 (m, 4H, cyclohex-4H + -5H). LRMS: MS (ES^+) *m/z* =173.7 $(M + 1)^+$.

4-Phenylethynyl-1*H*-imidazole trifluoroacetate (19h)

Yield 50%. Conversion >95%. ^{1}H-NMR ($CDCl_3$): δ 8.48 (s, 1H, imidazole-2H), 7.50-7.15 (m, 6H, imidazole-5H + Ph-H). LRMS: MS (ES^+) *m/z* =169.7 $(M + 1)^+$.

4-*p*-Tolylethynyl-1*H*-imidazole trifluoroacetate (19i)

Yield 62%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.31 (s, 1H, imidazole-2H), 7.35-7.22 (m, 3H, imidazole-5H + 2,6-phenyl-H), 7.10 (d, 2H, 3,5-phenyl-H), 2.30 (s, 3H, *p*-CH_3). LRMS: MS (ES^+) *m/z* =183.7 $(M + 1)^+$.

4-(3-Methoxy-phenylethynyl)-1*H*-imidazole trifluoroacetate (19j)

Yield 54%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.15 (s, 1H, imidazole-2H), 7.30-6.80 (m, 5H, imidazole-5H + Ph-H), 4.25 (s, 3H, OCH_3). LRMS: MS (ES^+) *m/z* =199.7 $(M + 1)^+$.

4-(4-Methoxy-phenylethynyl)-1*H*-imidazole trifluoroacetate (19k)

Yield 55%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.50 (s, 1H, imidazole-2H), 7.92 (d, 2H, 3,5-phenyl-H), 7.22 (s, 1H, imidazole-5H), 6.88 (d, 2H, 2,6-phenyl-H), 3.79 (s, 3H, OCH_3). LRMS: MS (ES^+) *m/z* =217.8 $(M + 1)^+$.

4-(3-Trifluoromethyl-phenylethynyl)-1*H*-imidazole trifluoroacetate (19l)

Yield 48%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.25 (s, 1H, imidazole-2H), 7.70-7.25 (m, 5H, imidazole-5H + Ph-H). LRMS: MS (ES^+) *m/z* =237.6 $(M + 1)^+$.

4-(4-Trifluoromethyl-phenylethynyl)-1*H*-imidazole trifluoroacetate (19m)

Yield 45%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.08 (s, 1H, im-2H), 7.55 (s, 4H, Ph), 7.35 (s, 1H, im-5H). LRMS: MS (ES^+) *m/z* =237.5 $(M + 1)^+$.

4-(2-Fluoro-phenylethynyl)-1*H*-imidazole trifluoroacetate (19n)

Yield 45%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 7.95 (s, 1H, imidazole-2H), 7.45-7.20 (m, 3H, imidazole-5H + 3,4-phenyl-H), 7.03 (m, 2H, 5,6-phenyl-H). LRMS: MS (ES^+) *m/z* =187.6 $(M + 1)^+$.

4-(4-Fluoro-phenylethynyl)-1*H*-imidazole trifluoroacetate (19o)

Yield 48%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.15 (s, 1H, imidazole-2H), 7.45-7.25 (m, 3H, imidazole-5H + 2,6-phenyl-H), 6.95 (t, 2H, 3,5-phenyl-H). LRMS: MS (ES^+) *m/z* =187.6 $(M + 1)^+$.

4-(2,4-Difluoro-phenylethynyl)-1*H*-imidazole trifluoroacetate (19p)

Yield 41%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.00 (s, 1H, imidazole-2H), 8.48-7.25 (m, 3H, imidazole-5H + 6-phenyl-H), 6.80 (m, 2H, 3,5-phenyl-H). LRMS: MS (ES^+) *m/z* =205.6 $(M + 1)^+$.

4-(2-Chloro-phenylethynyl)-1*H*-imidazole trifluoroacetate (19q)

Yield 42%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 7.85 (s, 1H, imidazole-2H), 7.45-7.10 (m, 5H, imidazole-5H + Ph-H). LRMS: MS (ES^+) *m/z* =203.8 $(M + 1)^+$.

4-(4-Chloro-phenylethynyl)-1*H*-imidazole trifluoroacetate (19r)

Yield 54%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.45 (s, 1H, imidazole-2H), 7.39-7.20 (m, 5H, imidazole-5H + Ph-H). LRMS: MS (ES^+) *m/z* =203.8 $(M + 1)^+$.

3-(1*H*-Imidazol-4-ylethynyl)-phenylamine ditrifluoroacetate (19s)

Yield 37%. Conversion 83%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.55 (s, 1H, imidazole-2H), 7.45-6.80 (m, 5H, imidazole-5H + Ph-H). LRMS: MS (ES^+) *m/z* =184.7 $(M + 1)^+$.

4-(1*H*-Imidazol-4-ylethynyl)-phenylamine ditrifluoroacetate (19t)

Yield 37%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.55 (d, 1H, imidazole-2H), 7.45-6.80 (m, 5H, imidazole-5H + Ph-H). LRMS: MS (ES^+) *m/z* =202.8 $(M + 1)^+$.

4-Naphtalen-1-ylethynyl-1*H*-imidazole trifluoroacetate (19u)

Yield 58%. Conversion. >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.35 (d, 1H, napht-2H), 8.30 (s, 1H, imidazole-2H), 7.80-7.30 (m, 7H, imidazole-5H + napht-H). LRMS: MS (ES^+) *m/z* =219.6 $(M + 1)^+$.

3-(1*H*-Imidazol-4-ylethynyl)pyridine trifluoroacetate (19v)

Yield 45%. Conversion >95%. ^{1}H-NMR ($CDCl_3$/MeOD): δ 8.65 (s, 1H, imidazole-2H), 8.53 (m, 2H, pyr-2,6-H), 7.82 (d, 1H, pyr-4H), 7.46 (s, 1H, imidazole-5H), 7.36 (dd, 1H, pyr-5H). LRMS: MS (ES^+) *m/z* =170.7 $(M + 1)^+$.

Solution-Phase Synthesis

4-(Pent-1-ynyl)-1*H*-imidazole oxalate(19a)

A mixture of **16**[23] (300 mg, 1.55 mmol), 10 mol % CuI (29.5 mg, 0.16 mmol), 5 mol % $Pd(Ph_3P)_4$ (89.6 mg, 0.078 mmol) in degassed NMP and degassed Et_3N (2.15 mL, 15.5 mmol) was added with 1-pentyne (0.23 mL, 2.33 mmol) and heated at 80 °C. After 3 h the mixture was cooled, diluted with Et_2O (30 mL) and washed with H_2O (3 x 10 mL). The organics were dried ($MgSO_4$), concentrated and purified by flash chromatography switching gradually the eluent from ethyl acetate/hexane (2/8) into ethyl acetate to afford 33 mg of product. The free base was converted into the oxalate salt, using the following procedure: the free base was dissolved in ethyl acetate with a few drops of methanol, then added dropwise with a solution of 1 equiv of oxalic acid dihydrate in ethyl acetate/methanol. The precipitate that formed was collected by filtration to afford 49 mg (14%) of a white powder. Mp: 189-190 °C (d). ^{1}H NMR (D_2O): δ 0.98 (t, 3H, CH_3), 1.55 (m, 2H, $CH_2CH_2CH_3$), 2.50 (t, 2H, $CCCH_2CH_2$), 7.45 (s, 1H, imidazole-5H), 8.46 (s, 1H, imidazole-2H). Anal. ($C_{10}H_{12}N_2O_4$) C, H, N.

4-(Hex-1-ynyl)-1*H*-imidazole oxalate(19b)

Using the same procedure as for **19a** with 300 mg (1.55 mmol) of **16**[23] and 0.27 mL (2.33 mmol) of 1-hexyne yielded 88 mg (24%) of product. White powder. Mp: 184-185 °C. ^{1}H NMR (D_2O): δ 0.92 (t, 3H, CH_3), 1.31-1.55 (m, 4H, central CH_2's), 2.46 (t, 2H, $CCCH_2$), 7.47 (s, 1H, imidazole-5H), 8.49 (s, 1H, imidazole-2H). Anal. ($C_{11}H_{14}N_2O_4$) C, H, N.

4-(4-Trifluoromethyl-phenylethynyl)-1*H*-imidazole oxalate (19m)

Using the same procedure as for **19a** with 300 mg (1.55 mmol) of **16**[23] and 0.38 mL (2.33 mmol) of 4-ethynyl-α,α,α-trifluorotoluene yielded 178 mg (35%) of product. White powder. Mp: 199.5-201.5 °C (d). ^{1}H NMR (CD_3OD): δ 7.73 (m, 5H, imidazole-5H + Ph-H), 8.39 (s, 1H, imidazole-2H). LRMS: MS (ES^+) *m/z* =237.3 $(M + 1)^+$. Anal. ($C_{14}H_9F_3N_2O_4$) C, H, N.

Table 4. Elemental analyses.

			C		H		N	
Cmpd	Code	Formula	Calc.	Found	Calc.	Found	Calc.	Found
19a	VUF 5908	$C_{10}H_{12}N_2O_4$	53.57	53.63	5.39	5.35	12.49	12.40
19b	VUF 5909	$C_{11}H_{14}N_2O_4$	55.46	55.25	5.92	5.81	11.76	12.00
19m	VUF 5924	$C_{14}H_9F_3N_2O_4$	51.54	51.26	2.78	2.53	8.58	8.84

Pharmacology

Radioligand Displacement Studies. Homogenates of SK-N-MC cells, stably expressing either the human histamine H_3[3] or the human histamine H_4 receptor,[17] were used for determining ligand affinities for the H_3 and H_4 receptor, respectively, as previously described.[58] Cell homogenates of H_3R-expressing cells (475 ± 32 fmol/mg of protein) were incubated for 40 min at 25 °C with 0.9-1.1 nM [^{3}H]-*N*$^\alpha$-methylhistamine (82 Ci/mmol) in 50 mM sodium phosphate buffer (pH 7.4) with or without competing ligands; cell homogenates of H_4R-expressing cells (620 ± 44 fmol/mg of protein) were incubated for 60 min at 37 °C with 9-11 nM [^{3}H]histamine (23.2 Ci/mmol) in 50 mM Tris HCl (pH 7.4), with or without competing ligands. Incubations were terminated by rapid dilution and subsequent filtration over Whatman GF/C filters pretreated with 0.3% polyethyleneimine using ice-cold wash buffer (H_3R: 25mM Tris HCl, 145 mM NaCl, pH 7.4 at 4 °C; H_4R: 50mM Tris HCl, pH 7.4 at 4 °C). The radioactivity retained on the filters was measured by liquid scintillation counting. Nonspecific binding was determined with 1 μM thioperamide as competing ligand. Competition isotherms were evaluated by a nonlinear, least squares curve-fitting using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Colorimetric cAMP Assay. Colorimetric cAMP assays were performed as previously described.[58] Briefly, SK-N-MC cells stably either the human histamine H_3[3] or the human histamine H_4 receptor,[17] and a cyclic AMP responsive element (CRE)-β-galactosidase reporter-gene were grown overnight in 96-well plates before the assay. To start the assay, the cells were incubated for 6 h with 1 μM forskolin and respective ligands at 37 °C. Thereafter, the medium was aspirated and cells were incubated overnight at 4 °C with 100 μL of assay buffer [100 mM NaH_2PO_4, 100 mM Na_2HPO_4, pH 8, 2 mM $MgSO_4$, 0.1 mM $MnCl_2$, 0.5% Triton, 40 mM β-mercaptoethanol, and 4 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG)]. The absorbance at 405 nm was determined by using a Victor^2 plate reader (Perkin-Elmer).

Analytical Methods. Protein levels were determined spectrophotometrically by a Packard Argus 400 microplate reader according to the method of Bradford,[59] using bovine serum albumin as a standard. All data shown are expressed as a mean ± S.E.M., and statistical

analyses were carried out by Student's t-test. P values < 0.05 were considered to indicate a significative difference.

Acknowledgments

The authors thank Dr. T. Lovenberg, Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA, for the cells stably expressing the human histamine H_3 and H_4 receptors.

References

(1) Hill, S. J.; Ganellin, C. R.; Timmerman, H.; Schwartz, J. C.; Shankley, N. P.; Young, J. M.; Schunack, W.; Levi, R.; Haas, H. L. International Union of Pharmacology. XIII. Classification of Histamine Receptors. *Pharmacol. Rev.* **1997**, *49*, 253-278.

(2) Leurs, R.; Smit, M. J.; Timmerman, H. Molecular Pharmacological Aspects of Histamine Receptors. *Pharmacol. Ther.* **1995**, *66*, 413-463.

(3) Lovenberg, T. W.; Roland, B. L.; Wilson, S. J.; Jiang, X.; Pyati, J.; Huvar, A.; Jackson, M. R.; Erlander, M. G. Cloning and Functional Expression of the Human Histamine H_3 Receptor. *Mol. Pharmacol.* **1999**, *55*, 1101-1107.

(4) Oda, T.; Morikawa, N.; Saito, Y.; Masuho, Y.; Matsumoto, S. I. Molecular Cloning and Characterization of a Novel Type of Histamine Receptor Preferentially Expressed in Leukocytes. *J. Biol. Chem.* **2000**, *275*, 36781-36786.

(5) Arrang, J. M.; Garbag, M.; Schwartz, J. C. Auto-inhibition of Brain Histamine Release Mediated by a Novel Class (H_3) of Histamine Receptors. *Nature Lond.* **1983**, *302*, 832-837.

(6) Blandina, P.; Giorgetti, M.; Bartolini, L.; Cecchi, M.; Timmerman, H.; Leurs, R.; Pepeu, G.; Giovannini, M. G. Inhibition of Cortical Acetylcholine Release and Cognitive Performance by Histamine H_3 Receptor Activation in Rats. *Br. J. Pharmacol.* **1996**, *119*, 1656-1664.

(7) Garcia, M.; Floran, B.; Arias-Montano, J. A.; Young, J. M.; Aceves, J. Histamine H_3 Receptor Activation Selectively Inhibits Dopamine D_1 Receptor-Dependent [^{3}H]GABA Release from Depolarization-Stimulated Slices of Rat Substantia Nigra Pars Reticulata. *Neuroscience* **1997**, *80*, 241-249.

(8) Fink, K.; Schlicker, E.; Neise, A.; Gothert, M. Involvement of Presynaptic H_3 Receptors in the Inhibitory Effect of Histamine on Serotonin Release in the Rat Brain Cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1990**, *342*, 513-519.

(9) Celuch, S. M. Possible Participation of Histamine H_3 Receptors in the Modulation of Noradrenaline Release from Rat Spinal Cord Slices. *Eur. J. Pharmacol.* **1995**, *287*, 127-133.

(10) Leurs, R.; Blandina, P.; Tedford, C.; Timmerman, H. Therapeutic Potential of Histamine H_3 Receptor Agonists and Antagonists. *Trends Pharm. Sci.* **1998**, *19*, 177-183.

(11) Schwartz, J. C.; Morisset, S.; Rouleau, A.; Tardivel-Lacombe, J.; Gbahou, F.; Ligneau, X.; Heron, A.; Sasse, A.; Stark, H.; Schunack, W.; Ganellin, C. R.; Arrang, J. M. Application of Genomics to Drug Design: The Example of the Histamine H_3 Receptor. *Eur. Neuropsychopharmacol.* **2001**, *11*, 441-448.

(12) Takahashi, K.; Suwa, H.; Ishikawa, T.; Kotani, H. Targeted Disruption of H_3 Receptors Results in Changes in Brain Histamine Tone Leading to an Obese Phenotype. *J. Clin. Invest.* **2002**, *110*, 1791-1799.

(13) Mignot, E.; Taheri, S.; Nishino, S. Emerging Therapeutic Targets for Sleep Disorders. *Nature Neurosci.* **2002**, *5*, 1071-1075.
(14) Miyazaki, S.; Imaizumi, M.; Onodera, K. Effects of Thioperamide, a Histamine H_3 Antagonist, on a Scopolamine-induced Learning Deficit Using an Elevated Plus-Maze Test in Mice. *Life Sci.* **1995**, *57*, 2137-2144.
(15) Onodera, K.; Miyazaki, S.; Imaizumi, M.; Stark, H.; Schunack, W. Improvement by FUB 181, a Novel Histamine H_3-Receptor Antagonist, of Learning and Memory in the Elevated Plus-maze Test in Mice. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *357*, 508-513.
(16) Morisset, S.; Traiffort, E.; Schwartz, J. C. Inhibition of Histamine Versus Acetylcholine Metabolism as a Mechanism of Tacrine Activity. *Eur. J. Pharmacol.* **1996**, *315*, R1-R2.
(17) Liu, C.; Ma, X. J.; Jiang, X.; Wilson, S. J.; Hofstra, C. L.; Blevitt, J.; Pyati, J.; Li, X.; Chai, W.; Carruthers, N.; Lovenberg, T. W. Cloning and Pharmacological Characterization of a Fourth Histamine Receptor (H_4) Expressed in Bone Marrow. *Mol. Pharmacol.* **2001**, *59*, 420-426.
(18) Hermkens, P. H. H.; Ottenheijm, H. C. J.; Rees, D. Solid-Phase Organic Reactions: A Review of the Recent Literature. *Tetrahedron* **1996**, *52*, 4527-4554.
(19) Terrett, N. K.; Gardner, M.; Gordon, D. W.; Kobylecki, R. J.; Steele, J. Combinatorial Synthesis-The Design of Compound Libraries and their Application to Drug Discovery. *Tetrahedron* **1995**, *51*, 8135-8173.
(20) Gordon, E. M.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gallop, M. A. Application of Combinatorial Technologies to Drug Discovery. 2. Combinatorial Organic Synthesis, Library Screening Strategies and Future Directions. *J. Med. Chem.* **1994**, *37*, 1385-1401.
(21) Lee, K.; Hwang, S. Y.; Park, C. W. Thrombin Inhibitors Based on a Propargylglycine Template. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1013-1018.
(22) Wright, J. L.; Gregory, T. F.; Boxer, P. A.; Meltzer, L. T.; Serpa, K. A.; Wise, L. D.; Hong-Bae, S.; Huang, J. C.; Konkoy, C. S.; Upasani, R. B.; Whittemore, E. R.; Woodward, R. M.; Yang, K. C.; Zhou, Z.-L. Discovery of Subtype-selective NMDA Receptor Ligands: 4-Benzyl-1-piperidinylalkynylpyrroles, Pyrazoles and Imidazoles as $NR1_A/2_B$ Antagonists. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2815-2818.
(23) Cliff, M. D.; Pyne, S. G. Synthesis of 4,4'-Bisimidazoles. *Synthesis* **1994**, 681-682.
(24) Kalinin, V. N. Carbon-Carbon Bond Formation in Heterocycles Using Ni- and Pd-Catalyzed Reactions. *Synthesis* **1992**, 413-432.
(25) Cliff, M. D.; Pyne, S. G. Palladium Catalysed Coupling of Imidazoles to Alkynyl and Vinyl Substrates. *Tetrahedron* **1996**, *52*, 13703-13712.
(26) Cliff, M. D.; Pyne, S. G. Asymmetric Synthesis of 2-Acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazoles. *J. Org. Chem.* **1997**, *62*, 1023-1032.
(27) Lindel, T.; Hochgürtel, M. Synthesis of the Marine Natural Product Oroidin and Its *Z*-Isomer. *J. Org. Chem.* **2000**, *65*, 2806-2809.
(28) Arrang, J. M.; Garbag, M.; Lancelot, J. C.; Lecomte, J. M.; Pollard, H.; Robba, M.; Schunack, W.; Schwartz, J. C. Highly Potent and Selective Ligands for Histamine H_3-Receptors. *Nature* **1987**, *327*, 117-123.
(29) Lipp, R.; Arrang, J. M.; Garbarg, M.; Luger, P.; Schwartz, J. C.; Schunack, W. Synthesis, Absolute Configuration, Stereoselectivity, and Receptor Selectivity of ($\alpha R,\beta S$)-α,β-Dimethylhistamine, a Novel Highly Potent Histamine H_3 Receptor Agonist. *J. Med. Chem.* **1992**, *35*, 4434-4441.
(30) Shih, N. Y.; Lupo, A. T. J.; Aslanian, R.; Orlando, S.; Piwinski, J. J.; Green, M. J.; Ganguly, A. K.; Clark, M. A.; Tozzi, S.; Kreutner, W.; Hey, J. A. A Novel Pyrrolidine Analog of Histamine as a Potent, Highly Selective Histamine H_3 Receptor Agonist. *J. Med. Chem.* **1995**, *38*, 1593-1599.
(31) De Esch, I. J. P.; Vollinga, R. C.; Goubitz, K.; Schenk, H.; Appelberg, U.; Hacksell, U.; Lemstra, S.; Zuiderveld, O. P.; Hoffmann, M.; Leurs, R.; Menge, W. M. P. B.; Timmerman, H. Characterization of the Binding Site of the Histamine H_3 Receptor. 1. Various Approaches to the Synthesis of 2-(1*H*-Imidazol-4-yl)cyclopropylamine and Histaminergic Activity of (1*R*,2*R*)- and (1*S*,2*S*)-2-(1*H*-Imidazol-4-yl)-cyclopropylamine. *J. Med. Chem.* **1999**, *42*, 1115-1122.

(32) Tedford, C. E.; Phillips, J. G.; Gregory, R.; Pawlowski, G. P.; Fadnis, L.; Khan, A.; Ali, S. M.; Handley, M. K.; Yates, S. L. Development of *trans*-2-[1*H*-Imidazol-4-yl] Cyclopropane Derivatives as New High-Affinity Histamine H_3 Receptor Ligands. *J. Pharm. Exp. Ther.* **1999**, *289*, 1160-1168.

(33) Ali, S. M.; Tedford, C. E.; Gregory, R.; Handley, M. K.; Yates, S. M.; Hirth, W. W.; Phillips, J. G. Design, Synthesis, and Structure-Activity Relationships of Acetylene-Based Histamine H_3 Receptor Antagonists. *J. Med. Chem.* **1999**, *42*, 903-909.

(34) Gliatech Inc. http://www.gliatech.com/news/news.cfm. **press release May 23, 2000**.

(35) Govoni, M.; Lim, H. D.; El-Atmioui, D.; Menge, W. M. P. B.; Bakker, R. A.; Timmerman, H.; Leurs, R. A Chemical Switch for the Modulation of the Functional Activity of Higher Homologs of Histamine on the Human Histamine H_3 Receptor: Effect of Various Substitutions at the Primary Amino Function. **to be published**.

(36) Nicolaou, K. C.; Winssinger, N.; Pastor, J.; Ninkovic, S.; Sarabia, F.; De, Y.; Vourloumis, D.; Yang, Z.; Li, T.; Giannakakou, P.; Hamel, E. Synthesis of Epothilones A and B in Solid and Solution Phase. *Nature* **1997**, *387*, 268-272.

(37) Marx, M. A.; Grillot, A.-L.; Louer, C. T.; Beaver, K. A.; Bartlett, P. A. Synthetic Design for Combinatorial Chemistry. Solution and Polymer-supported Synthesis of Polycyclic Lactams by Intramolecular of Azomethine Ylides. *J. Am. Chem. Soc.* **1997**, *119*, 6153-6167.

(38) Parikh, J. K.; Von E. Doering, W. Sulfur Trioxide in the Oxidation of Alcohols by Dimethyl Sulfoxide. *J. Am. Chem. Soc.* **1967**, *89*, 5505-5507.

(39) Rotella, D. P. Solid Phase Synthesis of Olefin and Hydroxyethylene Peptidomimetics. *J. Am. Chem. Soc.* **1996**, *118*, 12246-12247.

(40) Saha, A. K.; Liu, L.; Simoneaux, R. L.; Kukla, M. J.; Marichal, P.; Odds, F. Novel Antifungals Based on 4-Substituted Imidazole: A Combinatorial Chemistry Approach to Lead Discovery and Optimization. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2175-2178.

(41) Saha, A. K.; Liu, L.; Marichal, P.; Odds, F. Novel Antifungal Based on 4-Substituted Imidazole: Solid-Phase Synthesis of Substituted Aryl Sulfonamides Towards Optimization of In Vitro Activity. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2735-2739.

(42) Boojamra, C. G.; Burow, K. M.; Thompson, L. A.; Ellman, J. A. Solid-Phase Synthesis of 1,4-Benzodiazepine-2,5-diones. Library Preparation and Demonstration of Synthesis Generality. *J. Org. Chem.* **1997**, *62*, 1240-1256.

(43) Gelens, E.; Koot, W. J.; Menge, W. M. P. B.; Ottenheijm, H. C. J.; Timmerman, H. Solid-Phase Synthesis of 4-Substituted Imidazoles Using a Scaffold Approach. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1935-1938.

(44) Sonogashira, K.; Tohda, Y.; Hagihara, N. Direct Alkynylation of 5-iodo-1-methylimidazole proceeds in very Low Yields. *Tetrahedron Lett.* **1975**, *16*, 4467-4470.

(45) Stark, H.; Ligneau, X.; Arrang, J. M.; Schwartz, J. C.; Schunack, W. General Construction Pattern of Histamine H_3 Receptor Antagonists: Change of A Paradigm. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2011-2016.

(46) Yates, S. L.; Phillips, J. G.; Gregory, R.; Pawlowski, G. P.; Fadnis, L.; Khan, M. A.; Ali, S. M.; Tedford, C. E. Identification and Pharmacological Characterization of a Series of New 1*H*-4-Substituted-imidazoyl Histamine H_3 Receptor Ligands. *J. Pharm. Exp. Ther.* **1999**, *289*, 1151-1159.

(47) Ali, S. M.; Tedford, C. E.; Gregory, R.; Yates, S. L.; Phillips, J. G. New Acetylene Based Histamine H_3 Receptor Antagonists Derived from the Marine Natural Product Verongamine. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1133-1138.

(48) Liu, H.; Kerdesky, F. A.; Black, L. A.; Fitzgerald, M.; Henry, R.; Esbenshade, T. A.; Hancock, A. A.; Bennani, Y. L. An Efficient Multigram Synthesis of the Potent Histamine H_3 Antagonist GT-2331 and the Reassessment of the Absolute Configuration. *J. Org. Chem.* **2004**, *69*, 192-194.

(49) Tedford, C. E.; Hoffmann, M.; Seyedi, N.; Marruyama, R.; Levi, R.; Yates, S. L.; Ali, S. M.; Phillips, J. G. High Antagonistic Potency of GT-2227 and GT-2331, New Histamine H_3 Receptor Antagonists, in Two Functional Models. *Eur. J. Pharmacol.* **1998**, *351*, 307-311.

(50) Fox, G. B.; Bao Pan, J.; Esbenshade, T. A.; Bennani, Y. L.; Black, L. A.; Faghih, R.; Hancock, A. A.; Decker, M. W. Effects of Histamine H_3 Receptor Ligands GT-2331 and

Cyproxifan in a Repeated Acquisition Avoidance Response in the Spontaneously Hypertensive Rat Pup. *Behav. Brain Res.* **2002**, *131*, 151-161.

(51) Iddon, B.; Ngochindo, R. I. Synthesis and Reactions of Lithiated Monocycles Azoles containing 2 or more heteroatoms. 4. Imidazoles. *Heterocycles* **1994**, *38*, 2487-2568.

(52) Turner, R. M.; Lindell, S. D.; Ley, S. V. A Facile Route to Imdazol-4-yl Anions and their Reaction with Carbonyl Compounds. *J. Org. Chem.* **1991**, *56*, 5739-5740.

(53) Turner, R. M.; Ley, S. V.; Lindell, S. D. Synthesis of 4-Substituted Imidazoles via 4-Metallo Imidazole Intermediate. *Synlett.* **1993**, *10*, 748-750.

(54) Diederich, F.; Stang, P. J. *Metal-catalyzed Cross-coupling Reactions.*; Wiley-VCH Verlag GmbH: Weinhein, Germany, 1998; 214.

(55) Cliff, M. D.; Pyne, S. G. Asymmetric Synthesis of 2-Acetyl-4(5)-(1,2,4-trihydroxybutyl)imidazoles. *J. Org. Chem.* **1995**, *60*, 2378-2383.

(56) Cliff, M. D.; Pyne, S. G. Asymmetric Synthesis of (1*R*,2*S*,3*R*)-2-Acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole. *Tetrahedron Lett.* **1995**, *36*, 5969-5972.

(57) Lindel, T.; Hochgürtel, M. The Alkyne Pathway to Keramadine from the Marine Sponge *Agelas* sp. *Tetrahedron Lett.* **1998**, *39*, 2541-2544.

(58) Kitbunnadaj, R.; Zuiderveld, O. P.; De Esch, I. J. P.; Vollinga, R. C.; Bakker, R. A.; Lutz, M.; Spek, A. L.; Cavoy, E.; Deltent, M.-F.; Menge, W. M. P. B.; Timmerman, H.; Leurs, R. Synthesis and Structure-Activity Relationships of Conformationally Constrained Histamine H_3 Receptor Agonists. *J. Med. Chem.* **2003**, *46*, 5445-5457.

(59) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Anal. Biochem.* **1976**, *72*, 248-254.

Chapter 7

Attempts towards the synthesis of fluorescent conjugates for the histamine H_3 receptor.

Marinella Govoni,[a] Barrie Kellam,[b] Tim Self,[c] Stephen J. Hill,[c] Henk Timmerman[a] and Rob Leurs[a]

[a] Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV, Amsterdam, The Netherlands.

[b] School of Pharmaceutical Sciences, University Park, Nottingham NG7 2RD, UK.

[c] Institute of Cell Signalling, Medical School, Queen's Medical Center, University of Nottingham, Nottingham NG7 2UH, United Kingdom.

Abstract

The histamine H_3 receptor (H_3R) is thought to play a role in a number of diseases and CNS disorders and has consequently been recognized as a valuable target for the design of new drugs. Fluorescent ligands combined with confocal microscopy have proven to be a suitable new method for studying receptors and receptor-mediated mechanisms. The use of high-affinity fluorescent probes for the H_3R could thus help in elucidating molecular characteristics, regional distribution and cellular localization of this receptor. Here, we report our attempts towards the synthesis of fluorescent conjugates for the H_3R. Imbutamine and impentamine, two well-known H_3R ligands, were derivatised with six fluorescent moieties, dansyl, NBD, diethylamino-coumarine, BODIPY® 630/650-X, Cy3B™ and Alexa Fluor® 660. The synthesised molecules were first evaluated for their purity and stability and three of them, namely impentamine-dansyl **3**, impentamine-NBD **4** and imbutamine-diethylaminocoumarine **5**, were then assessed in binding assays on the human H_3R as well as for their fluorescence properties. Moreover, compounds **3-5** were also used in confocal microscopy experiments in an attempt to label human H_3R in live cells and therefore determine their sub-cellular localization. Conjugate **5** was found to be a high-affinity H_3R ligand (pK_i=8.7), but failed to label the hH_3R expressed in CHO cells.

Introduction

The histamine H_3 receptor (H_3R),[1,2] a member of the large family of seven transmembrane-spanning G-protein coupled receptors, is predominantly expressed in the brain.[3] The H_3R has been shown to mediate the inhibition of synthesis and release of histamine from histaminergic neurons via a negative feedback loop, but also to modulate the release of other neurotransmitters, such as acetylcholine,[4,5] dopamine,[6,7] serotonin[8,9] and noradrenaline,[10,11] in both the central and peripheral nervous systems. The H_3R seems to be implicated in a number of diseases and it has consequently been recognized as a valuable target for the design of new drugs.[12] H_3R ligands have indeed been proposed to be potential drugs for the treatment of many diseases and CNS disorders, such as attention-deficit hyperactivity disorder (ADHD),[13,14] Alzheimer's disease,[15] epilepsy,[16,17] schizophrenia,[18,19] obesity,[20,21] myocardial ischemia,[22] migraines and sleep disorders.[23]

Fluorescent receptor ligands have proven to be useful tools for the investigation of the interactions of different receptors with their ligands in complement to classical methods such as radioligand binding and site-directed mutagenesis.[24-26] Indeed, such ligands offer a multiplicity of information such as the mechanism of ligand binding,[27,28] the localization, movement and internalisation of receptors in living cells,[29,30] the distances between ligands and fluorescently labeled amino acids,[31,32] the physical nature of the binding pocket,[33,34] and the visualization of labeled receptors.[35,36] In addition, such ligands provide an attractive alternative to radioligands in receptor studies, circumventing several drawbacks associated with radioactivity such as high costs, potential health hazards and waste disposal problems.

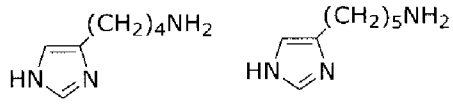
Due to its high sensitivity and speed, fluorescence microscopy is one of the most versatile techniques currently available for analyzing biomolecules and cells. The use of high-affinity molecular probes could thus help in elucidating the molecular characteristics of histamine receptor subtypes, their regional distribution and cellular localization.

Li and coworkers have recently reported the synthesis and pharmacological activity of fluorescent histamine H_1 and H_2 receptors antagonists related respectively to mepyramine[37] and potentidine,[38] but to our knowledge, no fluorescent ligands for the H_3R have been described to date in the literature. However, such fluorescent ligands should represent valuable tools for the visualization and the study of binding and activation mechanisms of the H_3R in different cell types and different species.

Here we report our attempts in the design, synthesis, physical and pharmacological characterization of the first fluorescent H_3R ligands. We also describe some attempts of visualization of fluorescent ligand-receptor complexes using confocal laser-scanning microscopy (CLSM) imaging techniques.

Design and synthesis of fluorescent ligands

The design of fluorescent conjugates of the H_3R was based on our previously reported ligands imbutamine and impentamine (respectively **1** and **2**, Figure 1), which show nanomolar affinity on the H_3R.[39-41]



Imbutamine **Impentamine**

Figure 1. Structures of 4-[1*H*-imidazol-4-yl]-butylamine (imbutamine) and 5-[1*H*-imidazol-4-yl]-pentylamine (impentamine).

For both compounds one point of attachment appeared chemically obvious: the primary amino function of the side chain. The N^{α} position of both **1** and **2** interacts with the aspartate residue Asp^{114} in transmembrane domain 3, which is highly conserved in the family of biogenic amines receptors.[42,43] As already discussed in Chapter 4, the primary amino function of the side chain can be modified with a high diversity of substituents, generating compounds spanning the whole spectrum of functional activities on the human H_3R.

We have thus directly linked the N^{α} position of imbutamine and impentamine with six known fluorescent moieties possessing different structures and fluorescence properties (compounds **3-8**, Schemes 1-3). The selected fluorophores, depicted in Figure 2, were: dansyl chloride (5-dimethylamino-naphthalene-1-sulfonyl chloride), NBD chloride (7-nitrobenz-2-oxa-1,3-diazol-4-yl chloride), 7-diethylaminocoumarin-3-carboxylic acid, BODIPY® 630/650-X, SE (6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl) amino hexanoic acid , succinimidyl ester), Cy3B™ NHS Ester and Alexa Fluor® 660 carboxylic acid, succinimidyl ester *6-isomer*.

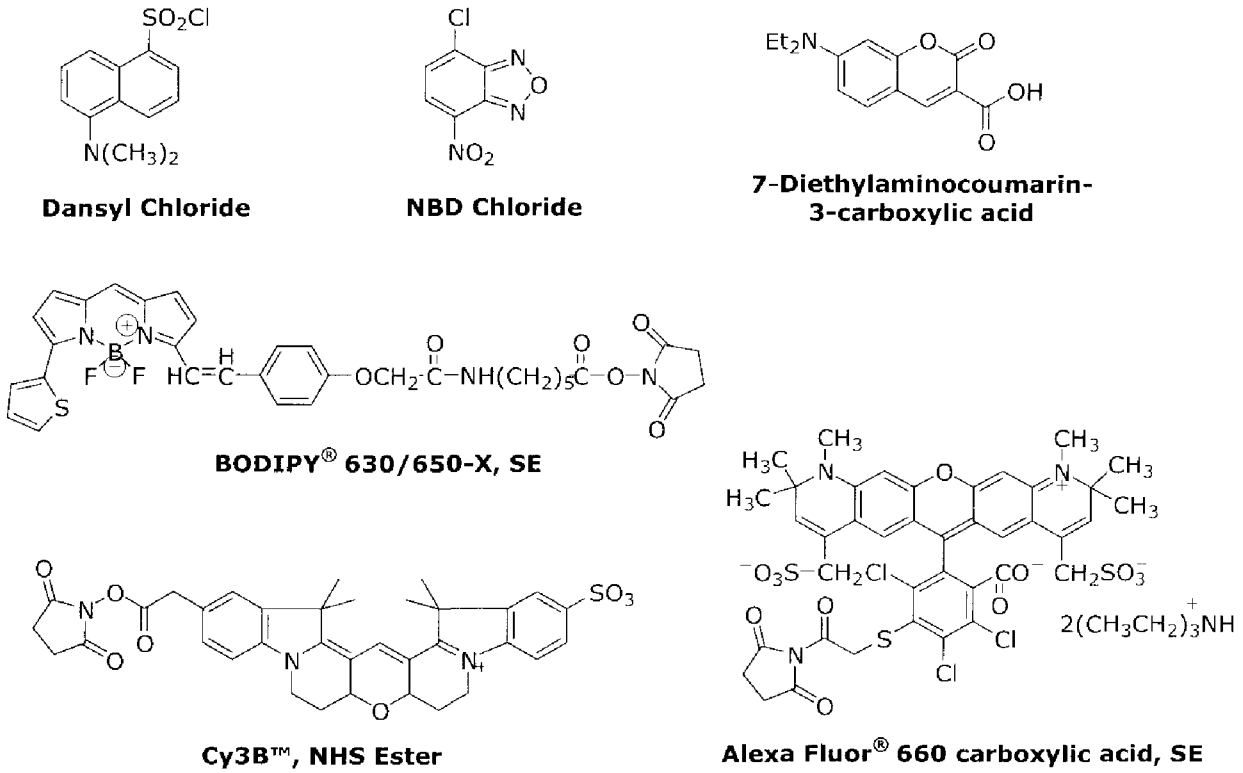
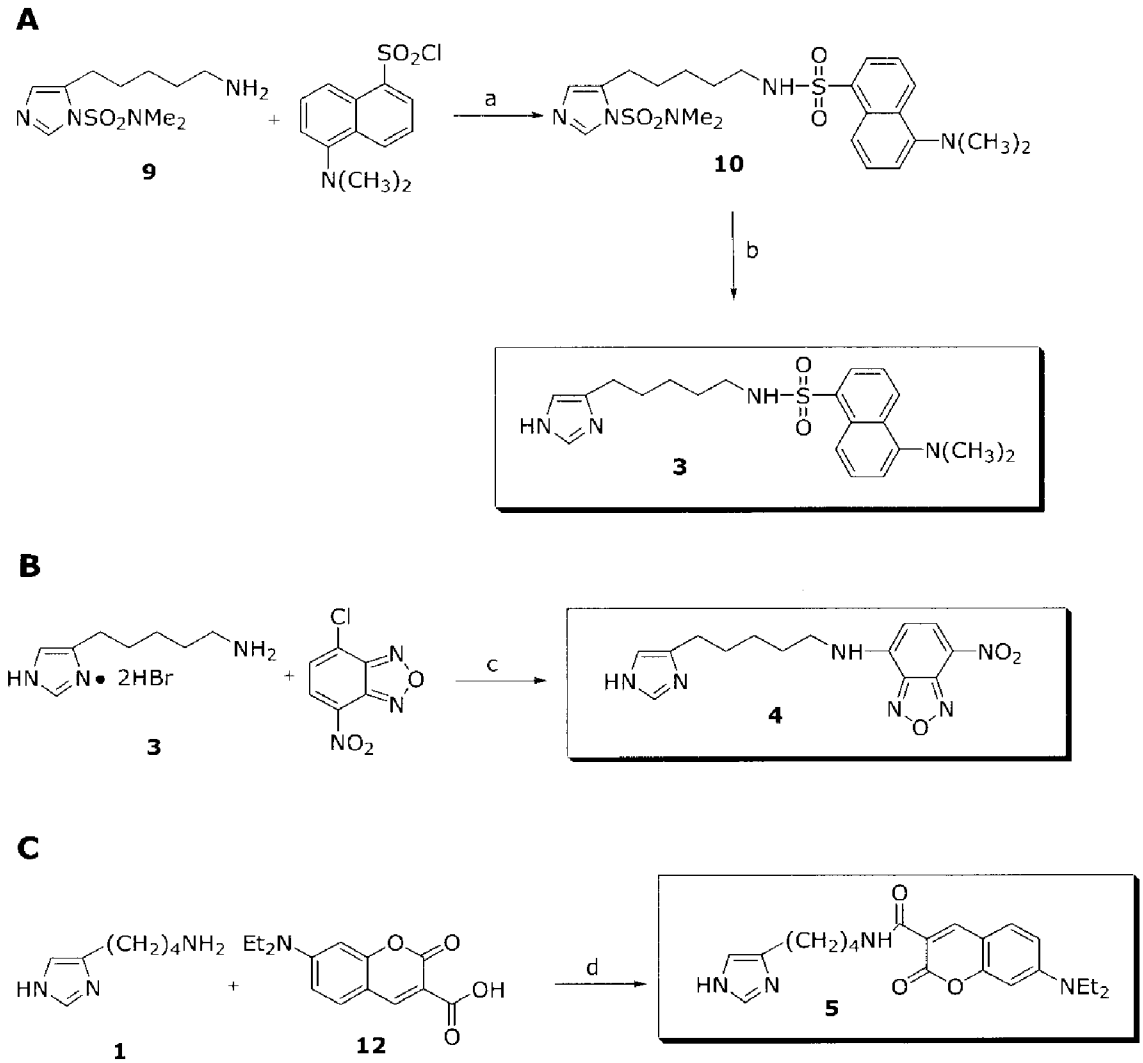


Figure 2. The fluorescent dyes used for coupling: dansyl chloride (5-dimethylamino-naphthalene-1-sulfonyl chloride), NBD chloride (7-nitrobenz-2-oxa-1,3-diazol-4-yl chloride), 7-diethylaminocoumarin-3-carboxylic acid, BODIPY® 630/650-X, SE (6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid, succinimidyl ester), Cy3B™ NHS Ester and Alexa Fluor® 610 carboxylic acid, succinimidyl ester *6-isomer*.

The first three, namely dansyl chloride, NBD chloride and 7-diethylaminocoumarin-3-carboxylic acid, were chosen because of their relatively small molecular volume in order to introduce a weak perturbation on the affinity. The latter three, e.g. BODIPY® 630/650-X, SE, Cy3B™ NHS Ester and Alexa Fluor® 660 carboxylic acid, SE, were instead selected because of their orange- to red-fluorescence properties, in addition to their high intensity fluorescence in the formed conjugates and good photostability. There are several major advantages of using fluorescent dyes that absorb in the red over those that absorb at shorter blue and green wavelengths. The most important of these advantages is the reduction in background that ultimately improves the sensitivity achievable; the number of fluorescent impurities is significantly reduced with longer excitation and emission wavelengths.[44-46] Excitation in the red spectral region is also advantageous when working with live cells because of reduced cell damage.[47] Moreover, the reactive dye BODIPY® 630/650-X, SE contains an additional seven atom aminohexanoyl spacer ("X") between the fluorophore and the succinimidyl ester group. This spacer helps to separate the fluorophore from its point of attachment, potentially reducing the interactions of the fluorophore with the molecule to which it is conjugated.[48]

Chemistry

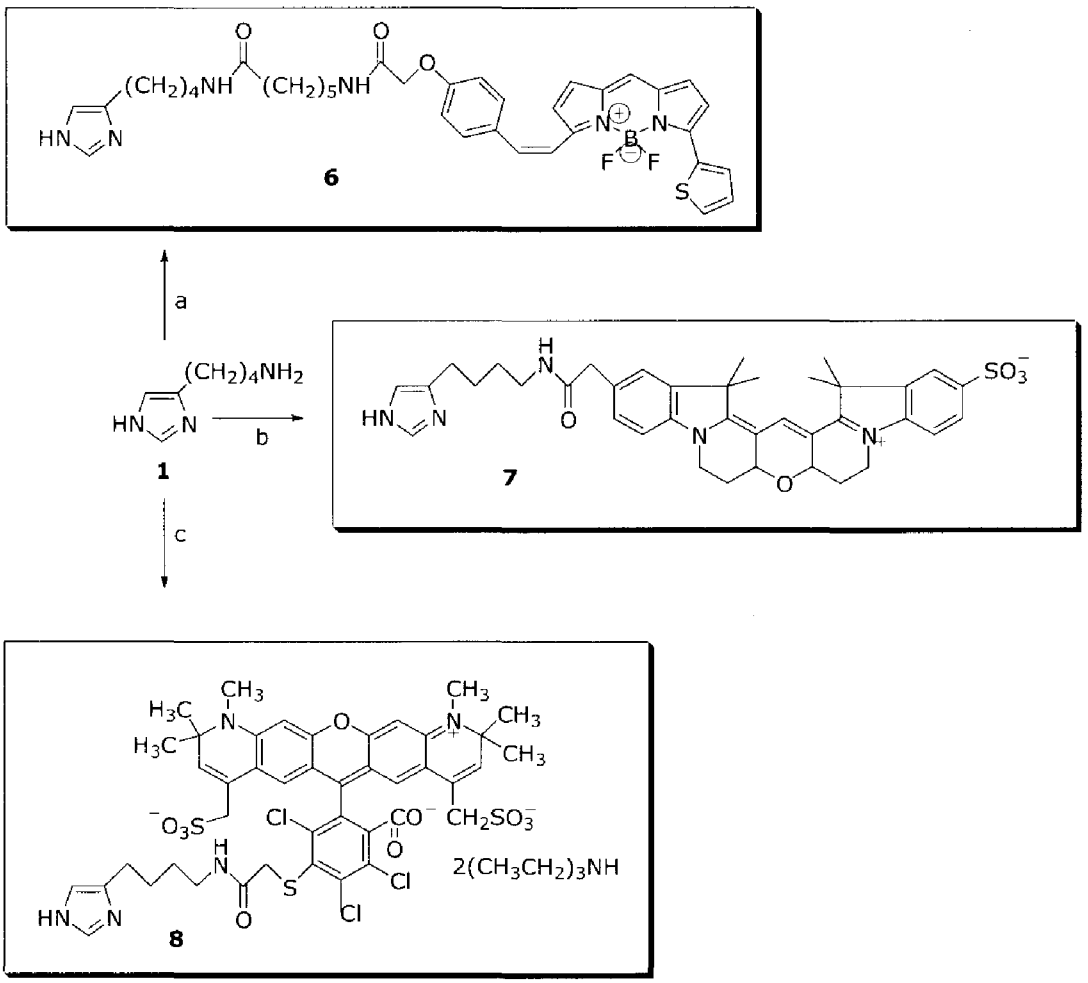
Compounds **3**, **4** and **5** were prepared as described in Scheme 1. Protected impentamine **9**[49] was reacted with dansyl chloride to give compound **10**, whose deprotection yielded conjugate **1** (Scheme 1A). Compound **4** was obtained by the direct linkage of the dihydrobromide salt of impentamine **2**[49] and NBD chloride, in the presence of triethylamine (TEA), which served the double function of "acid scavenger" base and of generating the free base of impentamine from the dihydrobromic salt (Scheme 1B). Grafting of imbutamine **1**[49] with coumarin derivative **12**[50] via an amide bond afforded compound **5** (Scheme 1C).



Scheme 1. Synthesis of compounds **3** (A), **4** (B) and **5** (C). Reagents: (a) Na_2CO_3, NaI, acetone, reflux; (b) 30% HBr, reflux; (c) TEA, MeOH, r.t.; (d) DCC, THF/DMF (1/1), 0 °C to r.t.

Conjugates **6-8** were prepared via acylation of the primary amino function of imbutamine **1**[49] with BODIPY® 630/650-X, SE, Cy3B™ NHS Ester and Alexa Fluor® 610 carboxylic acid, SE,

respectively, by stirring for 2 h in dry *N,N*-dimethylformamide (DMF) at room temperature, according to the synthetic pathway shown in Scheme 2.



Scheme 2. Synthesis of compounds **6-8**. Reagents: (a) BODIPY® 630/650-X SE, DMF, r.t.; (b) Cy3B™ NHS ester, DMF, r.t.; (c) Alexa Fluor® 660 SE, DMF, r.t.

Purification of compounds **6-8** was attempted either by (RP-C_{18})-preparative layer chromatography (PLC) or by preparative RP-HPLC on a C_{18} column using photodiode array detection, as described in the experimental section. The structure and purity of the conjugates were evaluated via ^{1}H-NMR spectroscopy and photodiode array analytical reverse phase HPLC.

Results and discussion

The H_3R affinity of the compounds was evaluated routinely by binding assays on the human H_3R, stably expressed in SK-N-MC cells,[2] using [^{3}H]-N$^\alpha$-methylhistamine as the radioligand (Table 1).

Table 1. Biological properties for the human H_3 receptor (hH_3R), fluorescence properties and confocal laser scanning microscopy (CLSM) of compounds **3-8** and of histamine-BODIPY® FL (HA-BY FL, Molecular Probes). The pk_i values are expressed as means ± SEM of separate experiments, each performed in triplicate.

compd (code)	pK_i hH_3R	λ_{abs} (nm)	λ_{em} (nm)	τ (ns)	Φ	CLSM
1 (VUF 4701)	8.4 ± 0.1	-[a]	-[a]	-[a]	-[a]	-[a]
2 (VUF 4702)	8.3 ± 0.1	-[a]	-[a]	-[a]	-[a]	-[a]
3 (VUF 5352)	6.7 ± 0.1	330	573	4	0.026	-[b]
4 (VUF 5354)	7.5 ± 0.1	482	563	11	0.013	-[b]
5 (VUF 5804)	8.7 ± 0.1	368	473	8	0.018	-[c]
6	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
7	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
8	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
HA-BY FL	6.1 ± 0.1	-[a]	-[a]	-[a]	-[a]	-[b]

[a] Not analyzed. [b] Compound rapidly internalised in the cell. [c] Not detectable.

Introduction of either dansyl- or NBD-moiety in the N'' position of compound **2**, leading to fluorescent conjugates **3** (impentamine-dansyl, Scheme 1A) and **4** (impentamine-NDB, Scheme 1B) causes a decrease of affinity on the H_3R, especially marked for ligand **3**. Introduction of a diethylaminocoumarine moiety on the primary amino function of **1** afforded derivative **5** (Scheme 1C). Binding data of **5** on the H_3R shows that despite the modification of the molecule to incorporate the fluorescent tag, it retains the properties required of a high affinity ligand (Table 1). More precisely, the H_3R affinity of ligand **5** was actually increased, with respect to the parent compound **1** (pk_i values of 8.7 and 8.4, respectively). Conjugate **5** presents one of the highest affinities for the H_3R, not only among this series of ligands, but also among the series of compounds already presented in chapter 4 (see Tables 1-3). Though showing lower H_3R affinities when compared to **5**, compounds **3** and **4** also retained a good affinity for the H_3R.

These results point to the fact that there is possibly a bulk tolerance on the receptor located around the basic amino group. Thus, a fluorescent group can be tolerated near this moiety.

Spectroscopic analyses of ligands **3-5** (Table 1) in degassed water solution showed that the excitation and emission spectra of the fluorophore remained virtually unchanged on coupling to imbutamine (**1**) or impentamine (**2**). Impentamine-dansyl (**3**) had excitation and emission maxima at 330 nm and 573 nm (Table 1), which were not significantly different from those for the dansyl-moiety itself (340 and 520 nm). For impentamine-NBD (**4**) excitation and emission maxima were 482 nm and 563 nm, compared to 465/535 nm of NDB alone. These values were 368 nm and 473 nm for imbutamine-diethylaminocoumarin (**5**), compared to 350/445 nm of the diethylaminocoumarin alone (Table 1).

The affinity values and the fluorescence properties of fluorescent conjugates **6-8** could not be evaluated, due to several problems encountered during the purification and confirmation of chemical identity of these compounds. Whilst the acylation reactions of **1** using the appropriate

fluorescent dye did not present any problems, it proved impossible to purify and conserve the afforded molecules **6-8** for an interval of time sufficient to carry out any further experimentation. Purification of derivatives **6-8**, immediately after evaporation of the reaction solvent, was attempted via several methods, namely preparative layer chromatography (PLC, compound **6**), RP-HPLC C_{18} semi-preparative column and PLC (**7**), and RP-C_{18}-PLC (silica gel, 1000 μm thickness, **8**), however none of these were successful. More precisely, conjugate imbutamine-BY630 (**6**) could be isolated in a form pure enough to allow us to confirm its identity via ^{1}H-NMR spectroscopy, but attempts toward the assessment of the purity by photodiode array analytical RP-HPLC failed, suggesting that the compound rapidly degraded immediately after purification by PLC. Fast degradation of compound **6** was also confirmed by thin layer chromatography (TLC); plates were run at several times after the last attempted purification and clearly showed a common degradation pathway, ultimately leading to complete decomposition of the initial molecule. We hypothesised that the instability of the conjugated molecule (**6**) could be due to the basicity of the imidazole ring and its "interference" with the relatively labile fluorophore moiety, leading in turn to its breakdown. In order to try confirming this supposition, we ran two parallel reactions; in the first one conjugation between benzylamine and BODIPY® 630/650-X (benzylamine-BY630) was attempted in dry DMF, whereas in the second one benzylamine-BY630 and imidazole were stirred together with BODIPY® 630/650-X in dry DMF. The reactions were followed at several times points via TLC. If our hypothesis had been true we should have observed different behaviors on TLC for the two reactions. Namely, we should have noticed a degradation pathway for the conjugate benzylamine-BY630 of the second reaction, possibly similar to the one observed for compound **6**, and no change for benzylamine-BY630 in the first reaction. In practice, we instead observed a similar degradation pathway for benzylamine-BY630 in both reactions, so our initial hypothesis could not be substantiated by experimental data.

Interestingly, a check on the purity of a one-year old frozen commercial batch of the conjugate histamine-BODIPY® FL (Molecular Probes), showed that the compound was partially degraded and the degradation pathway very much resembled the one observed for the synthesised conjugate **6**. Stability of histamine-BODIPY® FL was then tested by TLC in the same fashion described for compound **6** and indeed showed a common degradation pathway, also similar to the one observed for **6**.

Conjugates imbutamine-Cy3B (**7**) and imbutamine-Alexa Fluor 610 (**8**) were even more unstable; in fact, it was impossible to purify them in a form stable enough to allow us to assess their chemical identity via ^{1}H-NMR spectroscopy. However, TLCs were run at several times points after the last attempted purification, as already described for compound **6**, and again clearly showed a common degradation pathway, very similar to the one previously observed for imbutamine-BY630 (**6**), eventually leading to a complete disappearance of the initial molecule.

Thus, the rapid degradation of fluorescent conjugates **6-8**, either in solution or under dry conditions, prevented any further use of the compounds for evaluation of their biological properties and therefore their employment as fluorescent tools for the visualization of H_3R stably expressed in CHO cells in confocal microscopy imaging techniques.

Fluorescent labelling of CHO cells expressing the human H_3R was attempted with fluorescent conjugates **3-5**. Attempts toward the detection of the labelled receptors were realized using confocal microscopy (Figure 3).

In conventional microscopy, fluorescent images are degraded by light emitted or scattered by tissue that is out of the plane of focus. This produces an image of decreased resolution and reduced contrast. Confocal imaging, based on a principle developed by Minsky,[51] requires that illumination and detection systems are focused on the same small region within the tissue. Light from the illuminated volume is sampled so that the fluorescent signals arise from this area of tissue. Fluorescent signals that arise from outside the sampled area are "rejected" by a spatial filter, such as a pinhole. As both the illumination and detection systems are on the same focal plane, they are said to be confocal.[52,53]

Ligands **3-5** were used for the labelling of CHO cells expressing the hH_3R (50 fmol/mg of protein). The experiments with conjugates **3** and **5** were done at a λ_{exc} of 364 nm, whereas for compound **4** a λ_{exc} of 488 nm was used. Cells were incubated with the appropriate fluorescent conjugate and observed at 475 nm (**3** and **5**) or 505 nm (**4**).

In a first set of experiments CHO-H_3 cells were incubated with impentamine-dansyl (**3**, 100 nM, 10 min, 37 °C), and confocal images were captured (Figure 3). Figure 3A-1 shows a selected area before addition of the fluorescent conjugate, whereas in Figure 3A-2 an image of the same area taken at the end of the incubation time is depicted, clearly showing a diffuse fluorescence inside the cells due to the internalisation of ligand **3** in the cytoplasm. However, no fluorescence was detected on the perimeter of the cells, indicating that there was no formation of the receptor-ligand complex.

Similar observations were made when CHO-H_3 cells were incubated with impentamine-NBD (**4**, 100 nM, 10 min, 37 °C), and confocal images were captured (Figure 3B).

For visualization and detection of fluorescent ligand-receptor complexes on live cells it is essential that the fluorescent ligand is confined to the cell surface. Thus, retention in the extracellular compartment is advantageous for the binding of ligands to surface receptor sites.

In order to try maintaining conjugates **3** and **4** in the extracellular compartment, experiments were repeated either at lower concentrations (10 nM) or at lower temperature (room temperature), but unfortunately it proved impossible to avoid rapid diffusion of **3** and **4** into the intracellular compartment (data not shown). The lipophilic nature of those molecules caused them to enter intact cells, already at concentrations of 10 nM.

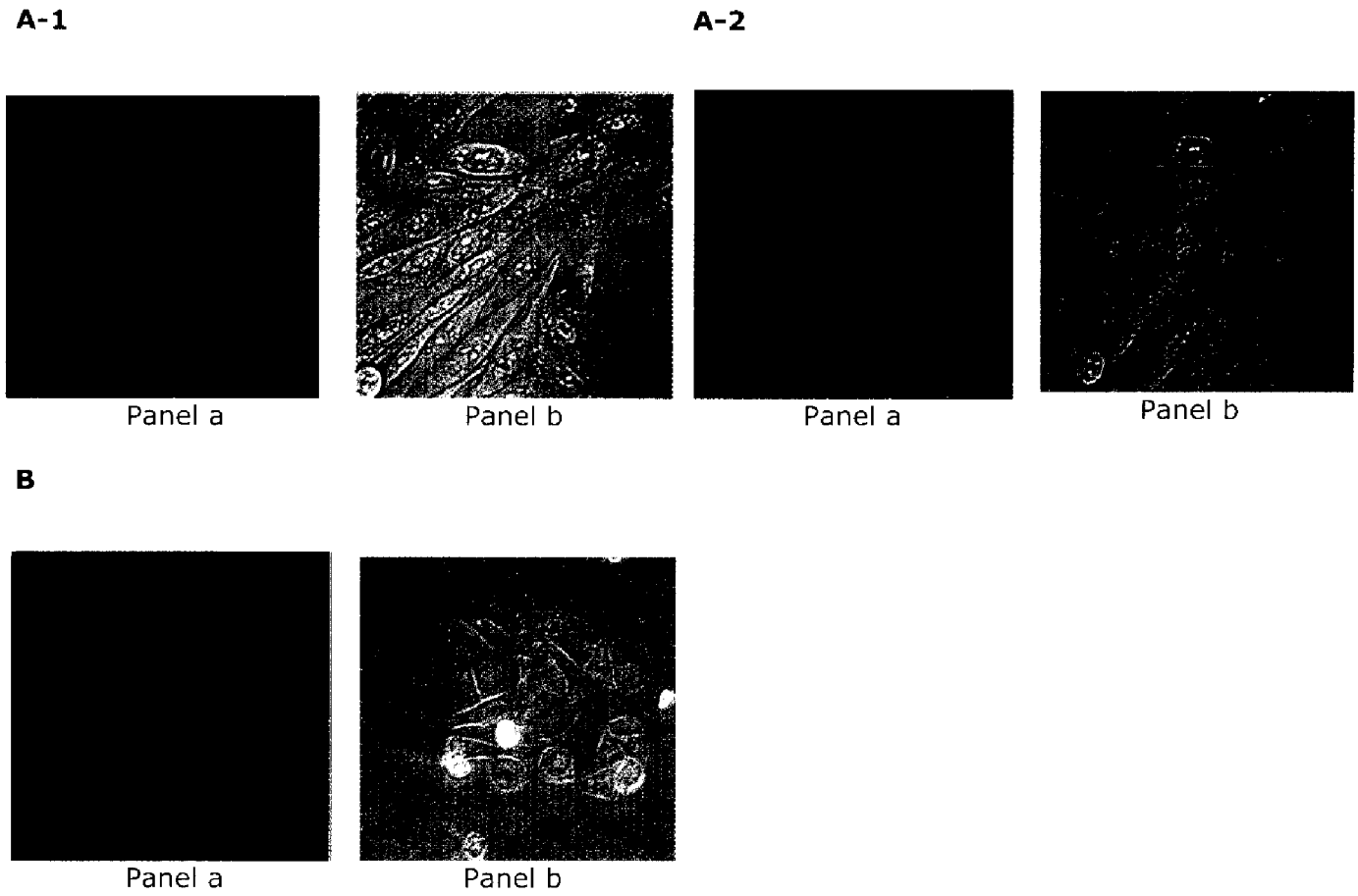


Figure 3. Fluorescent labelling studies of human H_3Rs in CHO cells. Visualization of impentamine- dansyl **3** (A) and impentamine-NBD **4** (B) in CHO-H_3 by confocal microscopy. A, CHO cells were incubated at 37 °C with 100 nM of **3** (A2) and without (A1). B, CHO cells were incubated at 37 °C with 100 nM of **4**. Panels b correspond to the same cells as panels a, observed in transmitted light.

The hydrophilic nature of the conjugate imbutamine-diethylaminocoumarine (**5**), combined with its high affinity for the H_3R (pK_i value of 8.7), led us to hypothesise that this molecule could be an ideal fluorescent ligand for labelling H_3Rs. However, unexpectedly, when CHO-H_3 cells were incubated with **5** (10 nM, 10 min, 37 °C), and confocal images were captured, no signs for the presence of the fluorescent molecule were detected anywhere in the cells, neither in the extracellular or intracellular compartments nor in the phospholipidic membrane (data not shown). This was very surprising, especially in consideration of the good fluorescence properties of the compound (Table 3). Experiments were then repeated with several combinations of different concentrations (10nM, 100 nM, 1μM and 10 μM), temperatures (37 °C and room temperature), incubation times (30 min, 1.5 h and 6 h) and laser potencies (20, 35 and 50%), but none of them enabled us to detect ligand **5** in the many confocal images captured (data not shown). We currently do not have an explanation available for the observed phenomenon, even though it could be hypothesised that the low density of the

heterologous receptor expression (50 fmol/mg of protein) might be one cause of the undetectability of compound **5**.

Additionally, an attempt of labelling H_3R-transfected CHO cells has been made with the commercially available histamine-BODIPY® FL. Despite the large fluorescent moiety linked to the terminal nitrogen of the side chain, the compound retains a reasonable affinity for the H_3R (pK_i=6.1, Table 1). This also confirms previous observations made for compounds **3-5**, pointing to the presence of a favourable region in the receptor located around the basic amino group. Confocal microscopy experiments were done at a λ_{exc} of 488 nm and the emission detected at 505 nm. The compound was incubated with CHO-H_3 cells (1μM; 10 min, r.t.) and confocal images were captured at several times during the incubation period. Immediately after injection of the fluorescent probe an increased fluorescence in the media surrounding the cells could be observed, which however disappeared almost instantly to reappear in the cytoplasm of the cells, thus highlighting an internalisation process of histamine-BODIPY FL® (data not shown).

Taken together, these results show that, besides good affinities, fluorescent ligands should also present appropriate physico-chemical properties, which are very important in determining whether a compound will be confined to the extracellular compartment or will enter the cytoplasmic area. However, even a good combination of physico-chemical, fluorescence and biological properties can sometimes still prevent the detection of a compound, and consequently prevent the use of such compound as fluorescent ligand in labelling experiments.

Conclusions

We have reported here our attempts to obtain potential fluorescent ligands for the study of the H_3R. However, despite our efforts we have so far not been able to synthesise a suitable fluorescent probe for the detection of H_3Rs in live cells.

Compounds **3-5** presented good (**3,4**) to excellent (**5**) H_3R affinities, but when used as fluorescent probes for the labelling of cells expressing the human H_3R, they were found to be unsuitable for the purpose. Compounds **3** and **4** were rapidly internalised in the cell cytoplasm, due to their high liposolubility, whereas compound **5**, despite its excellent H_3R affinity and better physico-chemical properties (lower liposolubility, compared to **3** and **4**), proved impossible to be detected in the cells via confocal microscopy, even when several different experimental conditions were used, e.g. concentrations, incubation times, temperatures and laser potencies.

On the other hand, ligands **6-8** turned out to be rapidly degrading, highly unstable compounds, which prevented their complete purification and therefore any further use as fluorescent tools in confocal microscopy experiments.

Though the hypothesis that the basicity of the imidazole ring might be the cause of the instability of compound **6** could not be substantiated, it might be an option trying to obtain fluorescent probes for the H_3R by modifying a suitable non-imidazolic H_3R ligand with the same fluorescent moiety as for compound **6**, e.g. BODIPY® 630/650-X. This fluorophore has already been used with success for derivatizing adenosine A_1 receptor (A_1R) ligands, such as xanthine amine congener (XAC) and N^6-aminobutyl adenosine (ABA), in order to obtain fluorescent probes for the A_1R.[54,55]

The ability to detect fluorescent ligand-receptor complexes on single cells and in sub-cellular compartments under physiological conditions is one of the big advantages of fluorescence imaging techniques over radioligand binding experiments. Fluorescent ligands combined with confocal microscopy have indeed been proven has a suitable new method for studying receptors and receptor-mediated mechanisms[26,56] in living tissues for a number of GPCRs, including α_1-adrenoceptors,[57] β_1- and β_2-adrenoceptors,[58] adenoside A_3 receptors[35] and 5-HT_4 receptors.[59]

However, the limiting step in the use of the aforementioned fluorescence-based methods for investigations on the H_3R, is so far the lack of stable, high affinity, hydrophilic fluorescent probes for this receptor. We have been unable to obtain such fluorescent-labelled H_3R ligands by connecting the primary amino group of imbutamine (**1**) or impentamine (**2**) with several blue- to red-fluorophores via sulphonamide (compound **3**) and carboxamide (**5**,**6-8**) bonds or arylation reaction (**4**).

Thus, new synthetic pathways and fluorescent conjugates for the achievement of appropriate fluorescent H_3R ligands will have to be investigated.

Experimental procedures

Chemistry

General procedures. Reagents were obtained from commercial suppliers and used without further purification. Solvents used were either AR or HPLC grade. Dry THF was freshly distilled from $LiAlH_4$.

Compounds **1**, **2** and **9** were obtained from our laboratory stock.[49]

Melting points were measured on an Electrothermal IA 9200 apparatus.

^1H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. Chemical shifts are given in ppm using the residual undeuterated solvent as reference.

Mass spectra were determined on a Perkin Elmer series 200 auto sampler.

Excitation and emission spectra and emission lifetime data were obtained by using an Edinburgh Instruments FLS920 fluorimeter.

Flash chromatography was performed on J.T.Baker Kieselgel 60.
All reactions were performed under an atmosphere of dry nitrogen and keeping the compounds protected from light.

5-[5-(5-Dimethylamino-naphthalene-1-sulfonylamino)-pentyl]-imidazole-1-sulfonic acid dimethylamide (10)

A solution of 5-(5-amino-pentyl)-imidazole-1-sulfonic acid dimethylamide **9**[49] (500 mg, 1.9 mmol) and dansyl chloride (5-dimethylamino-naphthalene-1-sulfonyl chloride) (460 mg, 1.7 mmol) in acetone (20 mL) were added with Na_2CO_3 (500 mg, 5 mmol) and NaI (300 mg, 1.7 mmol) and heated to reflux. After 3 h the solvent was removed in vacuo, the residue dissolved in water and extracted with ethyl acetate, then the organics were dried ($MgSO_4$) and evaporated to dryness. The product was purified by flash chromatography with ethyl acetate and then ethyl acetate/methanol (1/1) to afford 300 mg (33%) of product. ^{1}H NMR ($CDCl_3$): δ 1.27-1.52 (m, 6H, central CH_2's), 2.52 (t, 2H, imidazole-CH_2), 2.73-2.88 (m, 14H, $N(CH_3)_2$ (dansyl) + $N(CH_3)_2$ (protective group) + CH_2N), 6.65 (s, 1H, imidazole-4H), 7.11 (d, 1H, naphtyl), 7.48 (m, 2H, naphtyl), 7.79 (s, 1H, imidazole-2H), 8.19 (t, 2H, naphtyl), 8.48 (d, 1H, naphtyl).

5-Dimethylamino-naphthalene-1-sulfonic acid [5-(1*H*-imidazol-4-yl)-pentyl]-amide dioxalate (3)

Compound **10** (300 mg, 0.6 mmol) was dissolved in 30% HBr (50 mL) and heated under reflux. After 5 h the mixture was cooled and concentrated in vacuo, the residue was diluted with NaOH (20%) and extracted with $CHCl_3$. The organic layers were combined, dried ($MgSO_4$) and concentrated in vacuo to afford 160 mg (72%) of product. The free base was converted into the dioxalate salt, using the following procedure: the free base was dissolved in acetone, then added dropwise with a solution of 2 equiv of oxalic acid dihydrate in diethyl ether. The precipitate that formed was collected by filtration and washed with diethyl ether to afford 100 mg (41%) of a light purple powder. Mp: 115.5 °C (d). ^{1}H NMR (DMSO): δ 1.05-1.50 (m, 6H, central CH_2's), 2.40 (t, 2H, imidazole-CH_2), 2.73-2.90 (m, 8H, $N(CH_3)_2$ + CH_2N), 7.08 (s, 1H, imidazole-5H), 7.26 (d, 1H, naphtyl), 7.51-7.68 (m, 2H, naphtyl), 7.91 (t, 1H, naphtyl), 8.31 (d, 1H, naphtyl), 8.40-8.51 (m, 2H, imidazole-2H + naphtyl).

[5-(1*H*-Imidazol-4-yl)-pentyl]-(7-nitro-benzo[1,2,5]oxadiazol-4-yl)-amine dioxalate (4)

A solution of NBD chloride (320 mg, 1.6 mmol) in methanol (10 mL) was slowly added to a solution of 5-(1*H*-imidazol-4-yl)-pentylamine dihydrobromide **2**[49] (500 mg, 1.6 mmol) and triethylamine (0.82 mL, 8 mmol) in methanol (30 mL), then stirred at r.t. After 24 h the solvent was removed in vacuo, the residue dissolved in water and extracted with ethyl acetate,

then the organics were dried ($MgSO_4$) and evaporated to dryness. The product was purified by flash chromatography switching gradually the eluent from ethyl acetate to methanol to afford 250 mg (49%) of product. The free base was converted into a dioxalate as described for compound **3** yielding 185 mg (47%) of orange powder. Mp: 175.9-178.4 °C. ^{1}H NMR (D_2O): δ 1.32-1.82 (m, 6H, central CH_2's), 2.63 (t, 2H, imidazole-CH_2), 3.47-3.60 (m, 2H, CH_2N), 6.19 (d, 1H, NBD-5H), 7.11 (s, 1H, imidazole-5H), 8.31 (d, 1H, NBD-6H), 8.45 (s, 1H, imidazole-2H).

7-Diethylaminocoumarin-3-carboxylic acid [4-(1*H*-imidazol-4-yl)-butyl]-amide (5)

A solution of 7-diethylaminocoumarin-3-carboxylic acid[50] (430 mg, 1.65 mmol) in dry THF (50 mL) and dry DMF (50 mL) was added with 341 mg (1.65 mmol) of DCC at 0 °C. After 1 h the reaction mixture was allowed to warm to r.t., stirred for an additional hour, then added with a solution of imbutamine **1**[49] (230 mg, 1.65 mmol) in dry THF/dry DMF (1/1) (30 mL). After 24 h the mixture was filtered and the residue re-crystallized from hot isopropanol to afford 457 mg (72%) of product as a bright yellow powder. Mp: 139-140 °C. ^{1}H NMR ($CDCl_3/CD_3OD$): δ 1.12 (t, 6H, $(CH_3CH_2)_2$), 1.55 (m, 4H, central CH_2's), 2.48 (t, 2H, imidazole-CH_2), 2.78 (m, 2H, CH_2N), 3.32 (q, 4H, $(CH_3CH_2)_2$), 6.30 (s, 1H, chromene-4H), 6.50 (dd, 1H, chromene-5H), 6.58 (s, 1H, imidazole-5H), 7.25 (d, 1H, chromene-6H), 7,35 (s, 1H, imidazole, 2H), 8.25 (s, 1H,chromene-8H). LRMS: MS (ES^+) m/z =383.2 $(M + 1)^+$.

General Procedure for the synthesis of the coupled conjugates 6,7 and 8.

A solution of imbutamine **1**[49] (3 eq) in dry DMF was added with the appropriate fluorophore (1 eq) and stirred at r.t. After 2 h the solvent was removed in vacuo and the residue purified by PLC or HPLC.

Conjugate imbutamine-BODIPY® 630/650-X (6)

The compound was purified by three separate PLC runs in DCM/MeOH (80/20). R_f = 0.20. Blue powder. ^{1}H NMR (CD_3OD): δ 1.10-1.58 (m, 10H), 2.02 (t, 2H, CH_2ONH), 2.48 (t, 2H, imidazole-CH_2), 3.05 (t, 2H), 3.21 (m, 4H, CH_2NHCO), 4.48 (s, 1H, $NHCOCH_2O$), 6.63 (s, 1H, imidazole-5H), 6.72 (d, 1H), 6.90-7.18 (m, 6H), 7.25 (s, 1H), 7.40-7.58 (m, 6H), 8.02 (dd, 1H).

Conjugate imbutamine-Cy3B™ (7)

Purification attempted by RP-HPLC on a C_{18} column (Kromasyl 100-10 μm, 250 × 21 mm, pH 1-11) by using photodiode array detection (563 nm). Eluent: MeOH/ H2O/DIPA (25/75/0.3). Flow rate: 8 mL/min. t_R = 18.60 min. The solvents were removed in vacuo and the product was subjected to solid-phase extraction. Pink powder. Purification of a small batch of

compound was attempted by two separate PLC runs in DCM/MeOH (70/30). R_f = 0.25. Pink powder.

Conjugate imbutamine-Alexa Fluor® 660 (8)

Purification attempted by two separate RP-PLC (C_{18}) runs in CH_3CN/MeOH (80/20). Plates were pre-dried at 140 °C for 20 h. Each run was repeated three times. R_f = 0.30. Blue powder.

Biology

Radioligand Displacement Studies. Homogenates of SK-N-MC cells, stably expressing the human histamine H_3[2] were used for determining ligand affinities for the H_3 receptor, as previously described.[41] Cell homogenates of H_3R-expressing cells (131 ± 11 fmol/mg of protein) were incubated for 40 min at 25 °C with 0.9-1.1 nM [^{3}H]-N^α-methylhistamine (82 Ci/mmol) in 50 mM sodium phosphate buffer (pH 7.4) with or without competing ligands. Incubations were terminated by rapid dilution and subsequent filtration over Whatman GF/C filters pretreated with 0.3% polyethyleneimine using ice-cold wash buffer (25mM Tris HCl, 145 mM NaCl, pH 7.4 at 4 °C). The radioactivity retained on the filters was measured by liquid scintillation counting. Nonspecific binding was determined with 1 μM thioperamide as competing ligand. Competition isotherms were evaluated by a nonlinear, least squares curve-fitting using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Confocal microscopy. For live cell confocal microscopy, CHO cells stably expressing the human H_3R (50 fmol/mg of protein) were grown to 70% confluence in 35mm glass bottomed culture dishes (MatTek) in phenol red-free DMEM/F-12 media containing 10% (vol/vol) FCS and 2 mM glutamine. Cells were washed twice with sterile HEPES-buffered saline solution (HBS; 147 mM NaCl, 24 mM KCl, 1.3 mM $CaCl_2$, 1mM MgSO4, 1.5 mM $NaHCO_3$, 10 mM HEPES, 1mM sodium pyruvate, pH 7.4) and imaged either at room temperature (22 ± 2 °C) or at 37 °C on a heated microscope stage with an objective heater. Single confocal images were obtained on a Zeiss LSM510 confocal microscope using either a C-Apochromat 63 x 1.2W water immersion or a Plan Neofluar 40 x 1.3 numerical aperture oil-immersion objective lens. Cells were imaged in the continued presence of ligand (1024 x 1024 pixels; averaging four frames). Compound **3** and histamine BODIPY® FL were excited using 488nm Ar laser, with emission light collected through an LP505 emission filter. Compounds **4** and **5** were excited using 364-nm Ar laser, with emission light collected through an LP475 emission filter (**4**) or an LP505 emission filter (**5**).

Analytical Methods. Protein levels were determined spectrophotometrically by a Packard Argus 400 microplate reader according to the method of Bradford,[60] using bovine serum albumin as a standard. All data shown are expressed as a mean ± S.E.M., and statistical analyses were carried out by Student's t-test. P values < 0.05 were considered to indicate a significative difference.

Acknowledgments

The authors thank Ms. A. van der Stolpe for the synthesis of compounds **3** and **4**, and Mr. O.P. Zuiderveld for the radioligand displacement experiments of compounds **3-5** and histamine-BODIPY® FL.

References

(1) Arrang, J. M.; Garbag, M.; Schwartz, J. C. Auto-inhibition of Brain Histamine Release Mediated by a Novel Class (H_3) of Histamine Receptors. *Nature Lond.* **1983**, *302*, 832-837.

(2) Lovenberg, T. W.; Roland, B. L.; Wilson, S. J.; Jiang, X.; Pyati, J.; Huvar, A.; Jackson, M. R.; Erlander, M. G. Cloning and Functional Expression of the Human Histamine H_3 Receptor. *Mol. Pharmacol.* **1999**, *55*, 1101-1107.

(3) Lovenberg, T. W.; Pyati, J.; Chang, H.; Wilson, S. J.; Erlander, M. G. Cloning of Rat Histamine H_3 Receptor Reveal Distinct Species Pharmacological Profiles. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 771-778.

(4) Clapham, J.; Kilpatrick, G. J. Histamine H_3 Receptors Modulate the Release of [^{3}H]-Acetylcholine from Slices of Rat Entorhinal Cortex: Evidence for the Possible Existence of H_3 Receptor Sybtypes. *Br. J. Pharmacol.* **1992**, *107*, 919-923.

(5) Blandina, P.; Giorgetti, M.; Bartolini, L.; Cecchi, M.; Timmerman, H.; Leurs, R.; Pepeu, G.; Giovannini, M. G. Inhibition of Cortical Acetylcholine Release and Cognitive Performance by Histamine H_3 Receptor Activation in Rats. *Br. J. Pharmacol.* **1996**, *119*, 1656-1664.

(6) Schlicker, E.; Fink, K.; Detzner, M.; Gothert, M. Histamine Inhibits Dopamine Release in the Mouse Striatum via Presynaptic H_3 Receptors. *J. Neural Transm. Gen. Sect.* **1993**, *93*, 1-10.

(7) Garcia, M.; Floran, B.; Arias-Montano, J. A.; Young, J. M.; Aceves, J. Histamine H_3 Receptor Activation Selectively Inhibits Dopamine D_1 Receptor-Dependent [^{3}H]GABA Release from Depolarization-Stimulated Slices of Rat Substantia Nigra Pars Reticulata. *Neuroscience* **1997**, *80*, 241-249.

(8) Schlicker, E.; Betz, R.; Gothert, M. Histamine H_3 Receptor-mediated Inhibition of Serotonin Release in the Rat Brain Cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1988**, *337*, 588-590.

(9) Fink, K.; Schlicker, E.; Neise, A.; Gothert, M. Involvement of Presynaptic H_3 Receptors in the Inhibitory Effect of Histamine on Serotonin Release in the Rat Brain Cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1990**, *342*, 513-519.

(10) Schlicker, E.; Schunack, W.; Gothert, M. Histamine H_3 Receptor-mediated Inhibition of Noradrenaline Release in Pig Retina Discs. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1990**, *342*, 497-501.

(11) Celuch, S. M. Possible Participation of Histamine H_3 Receptors in the Modulation of Noradrenaline Release from Rat Spinal Cord Slices. *Eur. J. Pharmacol.* **1995**, *287*, 127-133.
(12) Leurs, R.; Blandina, P.; Tedford, C.; Timmerman, H. Therapeutic Potential of Histamine H_3 Receptor Agonists and Antagonists. *Trends Pharm. Sci.* **1998**, *19*, 177-183.
(13) Miyazaki, S.; Imaizumi, M.; Onodera, K. Effects of Thioperamide, a Histamine H_3 Antagonist, on a Scopolamine-induced Learning Deficit Using an Elevated Plus-Maze Test in Mice. *Life Sci.* **1995**, *57*, 2137-2144.
(14) Onodera, K.; Miyazaki, S.; Imaizumi, M.; Stark, H.; Schunack, W. Improvement by FUB 181, a Novel Histamine H_3-Receptor Antagonist, of Learning and Memory in the Elevated Plus-maze Test in Mice. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *357*, 508-513.
(15) Morisset, S.; Traiffort, E.; Schwartz, J. C. Inhibition of Histamine Versus Acetylcholine Metabolism as a Mechanism of Tacrine Activity. *Eur. J. Pharmacol.* **1996**, *315*, R1-R2.
(16) Yokoyama, H.; Onodera, K.; Iinuma, K.; Watanabe, T. Effect of Thioperamide, a Histamine H_3 Receptor Antagonist, on Electrically Induced Convulsions in Mice. *Eur. J. Pharmacol.* **1993**, *234*, 129-133.
(17) Schlicker, E.; Marr, I. The Moderate Affinity of Clozapine at H_3 Receptors is not shared by its two Major Metabolites and by Structurally Related and Unrelated Atypical Neuroleptics. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1996**, *353*, 290-294.
(18) Schwartz, J. C.; Morisset, S.; Rouleau, A.; Tardivel-Lacombe, J.; Gbahou, F.; Ligneau, X.; Heron, A.; Sasse, A.; Stark, H.; Schunack, W.; Ganellin, C. R.; Arrang, J. M. Application of Genomics to Drug Design: The Example of the Histamine H_3 Receptor. *Eur. Neuropsychopharmacol.* **2001**, *11*, 441-448.
(19) Pillot, C.; Ortiz, J.; Heron, A.; Ridray, S.; Schwartz, J.-C.; Arrang, J. M. Cyproxifan, a Histamine H_3 Antagonist/Inverse Agonist, potentiates Neurochemical and Behavioral Effects of Haloperidol in the Rat. *J. Neurosci.* **2002**, *22*, 7272-7280.
(20) Sakata, T.; Yoshimatsu, H.; Kurokawa, M. Hypothalamic Neuronal Histamine: Implications of its Homeostatic Control of Energy Metabolism. *Nutrition* **1997**, *13*, 403-411.
(21) Takahashi, K.; Suwa, H.; Ishikawa, T.; Kotani, H. Targeted Disruption of H_3 Receptors Results in Changes in Brain Histamine Tone Leading to an Obese Phenotype. *J. Clin. Invest.* **2002**, *110*, 1791-1799.
(22) Levi, R.; Smith, N. C. Histamine H_3 Receptors: A New Frontier in Myocardial Ischemia. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 825-830.
(23) Mignot, E.; Taheri, S.; Nishino, S. Emerging Therapeutic Targets for Sleep Disorders. *Nature Neurosci.* **2002**, *5*, 1071-1075.
(24) Baindur, N.; Triggle, D. J. Selective Fluorescent Ligands for Pharmacological Receptors. *Drug Dev. Res.* **1994**, *33*, 373-398.
(25) Baindur, N.; Triggle, D. J. Concept and Progress in the Development and Utilization of Receptor-specific Fluorescent Ligands. *Med. Res. Rev.* **1994**, *14*, 591-664.
(26) Mc Grath, J. C.; Arribas, S.; Daly, C. J. Fluorescent Ligands for the Study of Receptors. *Trends Pharm. Sci.* **1996**, *17*, 393-399.
(27) Tairi, A. P.; Hovius, R.; Pick, H.; Blasey, H.; Bernard, A.; Surprenant, A.; Lunström, K.; Vogel, H. Ligand Binding to the Serotonin 5-HT_3 Receptor Studied With a Novel Fluorescent Ligand. *Biochemistry* **1998**, *37*, 15850-15864.
(28) Madsen, B. W.; Beglan, C. L.; Spivak, C. E. Fluorescein-labeled Naloxone Binding to m Opioid Receptors on Live Chinese Hamster Ovary Cells Using Confocal Fluorescent Microscopy. *J. Neurosci. Methods* **2000**, *97*, 123-131.
(29) Beaudet, A.; Nouel, D.; Stroh, T.; Vandenbulcke, F.; Dal-Farra, C.; Vincent, J. P. Fluorescent Ligands for Studying Neuropeptide Receptors by Confocal Microscopy. *Braz. J. Med. Biol. Res.* **1998**, *31*, 1479-1489.
(30) Terillon, S.; Cheng, L. L.; Stoev, S.; Mouillac, B.; Barberis, C.; Manning, M.; Durroux, T. Synthesis and Characterization of Fluorescent Antagonists and Agonists for Human Oxytocin and Vasopressin V1a Receptors. *J. Med. Chem.* **2002**, *45*, 2579-2588.

(31) Valloton, P.; Tairi, A. P.; Wohland, T.; Friedrich-Benet, K.; Pick, H.; Hovius, R.; Vogel, H. Mapping the Antagonist Binding Site of the Serotonin Type 3 Receptor by Fluorescence Resonance Energy Transfer. *Biochemistry* **2001**, *40*, 12237-12242.

(32) Turcatti, G.; Nemeth, K.; Edgerton, M. D.; Knowles, J.; Vogel, H.; Chollet, A. Fluorescent Labeling of NK2 Receptor at Specific Sites in vivo and Fluorescence Energy Transfer Analysis of NK2 Ligand-receptor Complexes. *Receptor Channels* **1997**, *5*, 201-207.

(33) Turcatti, G.; Zoffmann, S.; Lowe, J. A.; Drozda, S. E.; Chassaing, G.; Schwartz, T. W.; Chollet, A. Characterization of Non-peptide Antagonist and Peptide Agonist Binding Sites of the NK1 Receptor with Fluorescent Ligands. *J. Biol. Chem.* **1997**, *272*, 21167-21175.

(34) Turcatti, G.; Nemeth, K.; Edgerton, M. D.; Meseth, U.; Talabot, F.; Peitsch, M.; Knowles, J.; Vogel, H.; Chollet, A. Probing the Structure and Function of the Tachykinin Neurokinin-2 Receptor Through Biosynthetic Incorporation of Fluorescent Amino Acids at Specific Sites. *J. Biol. Chem.* **1996**, *271*, 19991-19998.

(35) Macchia, M.; Salvetti, F.; Bertini, S.; Di Bussolo, V.; Gattuso, L.; Gesi, M.; Hamdan, M.; Klotz, K. N.; Laragione, T.; Lucacchini, A.; Minutolo, F.; Nencetti, S.; Papi, C.; Tuscano, D.; Martini, C. 7-Nitrobenzofurazan (NBD) Derivatives of 5'-*N*-Ethylcarboxamidoadenosine (NECA) as New Fluorescent Probes for Human A_3 Adenosine Receptors. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3023-3026.

(36) Hadrich, D.; Berthold, F.; Steckhan, E.; Bonisch, H. Synthesis and Characterization of Fluorescent Ligands for the Norepinephrine Transporter: Potential Neuroblastoma Imaging Agents. *J. Med. Chem.* **1999**, *42*, 3101-3108.

(37) Li, L.; Kracht, J.; Peng, S.; Bernhardt, G.; Buschauer, A. Synthesis and Pharmacological Activity of Fluorescent Histamine H_1 Receptor Antagonists Related to Mepyramine. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1245-1248.

(38) Li, L.; Kracht, J.; Peng, S.; Bernhardt, G.; Elz, S.; Buschauer, A. Synthesis and Pharmacological Activity of Fluorescent Histamine H_2 Receptor Antagonists Related to Potentidine. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1717-1720.

(39) Vollinga, R. C.; Menge, W. M. P. B.; Leurs, R.; Timmerman, H. Homologs of Histamine as Histamine H_3 Receptor Antagonists: A New Potent and Selective H_3 Antagonist, 4(5)-(5-Aminopentyl)-1*H*-imidazole. *J. Med. Chem.* **1995**, *38*, 266-271.

(40) Wieland, K.; Bongers, G.; Yamamoto, Y.; Hashimoto, T.; Yamatodani, A.; Menge, W. M. P. B.; Timmerman, H.; Lovenberg, T. W.; Leurs, R. Constitutive Activity of Histamine H_3 Receptors Stably Expressed in SK-N-MC Cells: Display of Agonism and Inverse Agonism by H_3 Antagonists. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 908-914.

(41) Kitbunnadaj, R.; Zuiderveld, O. P.; De Esch, I. J. P.; Vollinga, R. C.; Bakker, R. A.; Lutz, M.; Spek, A. L.; Cavoy, E.; Deltent, M.-F.; Menge, W. M. P. B.; Timmerman, H.; Leurs, R. Synthesis and Structure-Activity Relationships of Conformationally Constrained Histamine H_3 Receptor Agonists. *J. Med. Chem.* **2003**, *46*, 5445-5457.

(42) De Esch, I. J. P.; Timmerman, H.; Menge, W. M. P. B.; Nederkoorn, P. H. J. A Qualitative Model for the Histamine H_3 Receptor Explaining Agonistic and Antagonistic Activity Simultaneously. *Arch. Pharm. Pharm. Med. Chem.* **2000**, *333*, 254-260.

(43) Uveges, A. J.; Kowal, D.; Zhang, Y.; Spangler, T. B.; Dunlop, J.; Semus, S.; Jones, P. G. The Role of Transmembrane Helix 5 in Agonist Binding to the Human H_3 Receptor. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 451-458.

(44) Aubin, J. E. Autofluorescence of Viable Cultured Mammalian Cells. *J. Histochem. Cytochem.* **1979**, *27*, 35-43.

(45) Cheng, P. C.; Kriete, A. Image Contrast in Confocal Light Microscopy. *Handbook of Biological Confocal Microscopy*; 2nd ed.; Plenum Press: New York, 1995; pp 327-346.

(46) Affleck, R. L.; Ambrose, W. P.; Demas, J. N.; Goodwin, P. M.; Schecker, J. A.; Wu, M.; Keller, R. A. Reduction of Luminescent Background in Ultrasensitive Fluorescence Detection by Photobleaching. *Anal. Chem.* **1996**, *68*, 2270-2276.

(47) Terasaki, M.; Dailey, M. E. Confocal Microscopy on Living Cells. *Handbook of Biological Confocal Microscopy*; 2nd ed.; Plenum Press: New York, 1995; pp 327-346.

(48) Haugland, R. P. *Handbook of Fluorescent Probes and Reasearch Products*; Eight ed.; Molecular Probes: Eugene, 2001.

(49) Vollinga, R. C.; Menge, W. M. B. P.; Timmerman, H. A New Convenient Route for the Synthesis of 4(5)-(ω-Aminoalkyl)-1*H*-Imidazoles. *Recl. Trav. Chim. Pays-Bas* **1993**, *112*, 123-125.
(50) Corrie, J. E. T. Thiol-reactive Fluorescent Probes for Protein Labelling. *J. Chem. Soc. Perkin Trans. 1* **1994**, 2975-2982.
(51) Minsky, M. Microscopy Apparatus: US 3013467, 1961.
(52) Inoue, S. Foundations of Confocal Scanned Imaging in Light Microscopy. *Handbook of Biological Confocal Microscopy*; Plenum Press: New York, 1995; pp 1-17.
(53) Fine, A. Confocal Microscopy: Principle and Practice. *Imaging Neurons: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: New York, 1999; pp 11.11-11.11.
(54) Briddon, S. J.; Middleton, R. J.; Cordeaux, Y.; Flavin, F. M.; Weinstein, J. A.; George, M. W.; Kellam, B.; Hill, S. J. Quantitative Analysis of the Formation and Diffusion of A_1-Adenosine Receptor-Antagonist Complexes in Single Living Cells. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4673-4678.
(55) Briddon, S. J.; Middleton, R. J.; Yates, A. S.; George, M. W.; Kellam, B.; Hill, S. J. Application of Fluorescence Correlation Spectroscopy to the Measurement of Agonist Binding to a G-Protein Coupled Receptor at the Single Cell Level. *Faraday Discuss.* **2004**, *126*, 197-207.
(56) Daly, C. J.; Mc Grath, J. C. Fluorescent Ligands, Antibodies, and Proteins for the Study of Receptors. *Pharmacology and Therapeutics* **2003**, *100*, 101-118.
(57) Daly, C. J.; Milligan, C. M.; Milligan, G.; Mackenzie, J. F.; McGrath, J. C. Cellular Localization and Pharmacological Characterization of Functioning *Alpha*-1 Adrenoceptors by Fluorescent Ligand Binding and Image Analysis Reveals Identical Binding Properties of Clustered and Diffuse Populations of Receptors. *J. Exp. Pharm. Ther.* **1998**, *286*, 984-990.
(58) Baker, J. G.; Hall, I. P.; Hill, S. J. Pharmacology and Direct Visualization of BODIPY TMR-CGP: a Long Acting Fluorescent β_2-Adrenoceptor Antagonists. *Br. J. Pharmacol.* **2003**, *139*, 232-242.
(59) Berque-Bestel, I.; Soulier, J. L.; Giner, M.; Rivail, L.; Langlois, M.; Sicsic, S. Synthesis and Characterization of the First Fluorescent Antagonists for Human 5-HT_4 Receptors. *J. Med. Chem.* **2003**, *46*, 2606-2620.
(60) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Anal. Biochem.* **1976**, *72*, 248-254.

Appendix

Glassware and equipment used for solid phase reactions

In this appendix, the developed glassware and other equipment used for the solid phase synthesis of the imidazole containing compounds reported in chapter 6 are described.

Glassware

For the coupling reactions of starting materials to the resin a large glass reaction vessel (20 mL) was used (Figure 1), whereas for the modification of resin-bound imidazoles mostly small glass reaction vessels (5 mL) with a metal bottom were employed (Figure 1, MultiSynTech). To prevent corrosion by the TFA mixtures of metal parts, all-glass reactions vessels were used for the cleavage of the end-products (Figure 1).

Most reactions were performed under a nitrogen atmosphere. To this purpose the reaction vessels were placed in fitting outer vessels, which were capped with a septum. A nitrogen flow was applied via a needle through the septum (Figure 1). The nitrogen outlet was established in the same way. To exclude leakage the septa were usually used only once. If a reaction time of over 2 hours was necessary to complete the given reaction, the nitrogen flow was stopped to avoid evaporation of the solvent. A small positive nitrogen pressure was maintained instead.

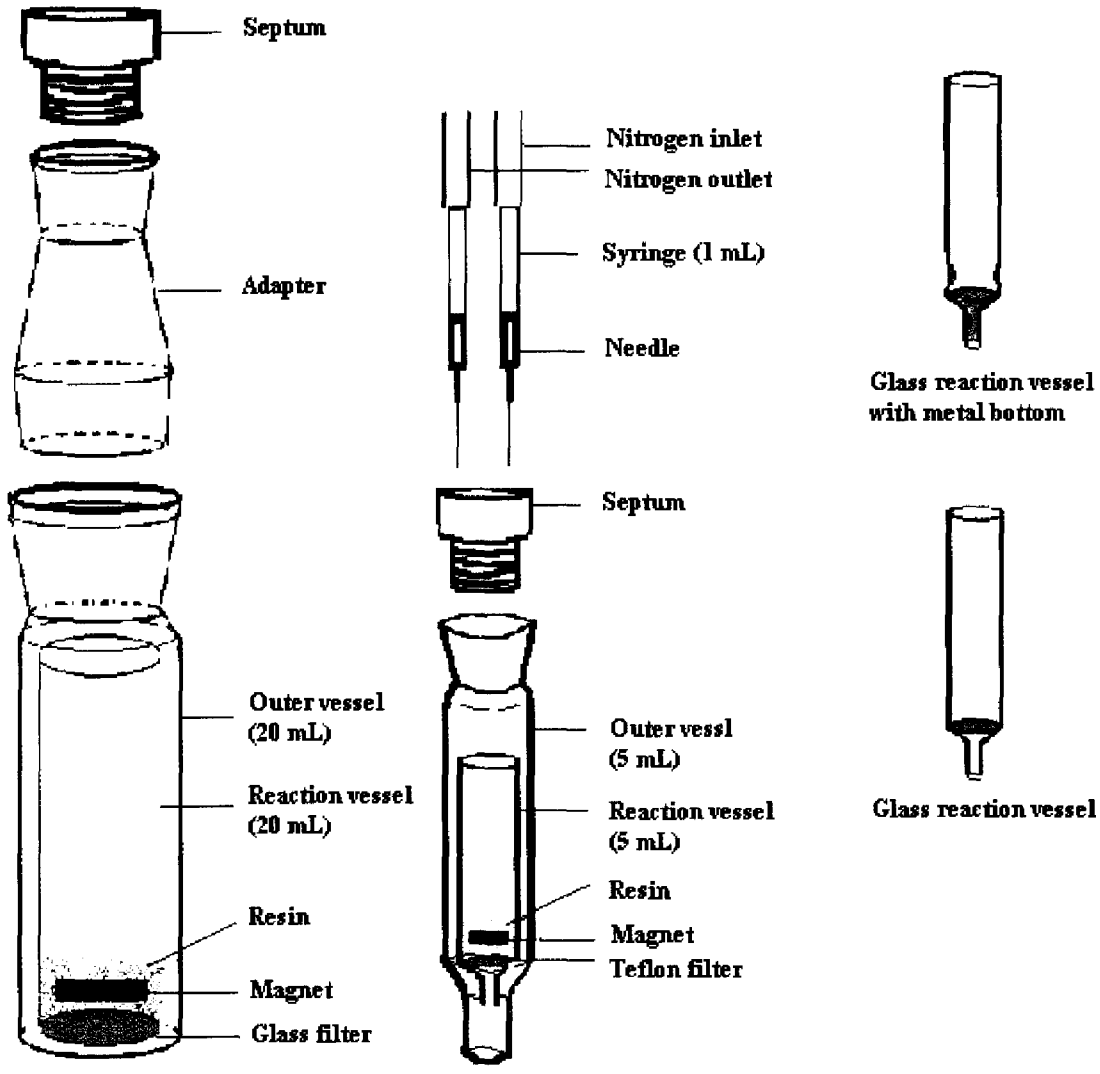


Figure 1. Glassware used for the solid-phase reactions.

Heating of reaction mixtures

For some reactions heating of the reaction mixture was necessary. For that purpose, the equipment shown in Figure 2 was developed. In a cylindrical slab of aluminium with an indent in the bottom to fit over a common laboratory hot plate with magnetic stirrer, eight holes were drilled to hold the reaction vessels. To monitor the temperature, a thermometer was placed in the small hole in the middle of the unit. With this apparatus, 8 reactions can be performed at the same time. To perform reactions, outer vessels (with reaction vessels inside) are placed in the heating/stirring unit. Condensers are put on top and a flow of nitrogen (or argon) and/or vacuum can be established via the capped nitrogen inlet (connected to a vacuum /nitrogen manifold).

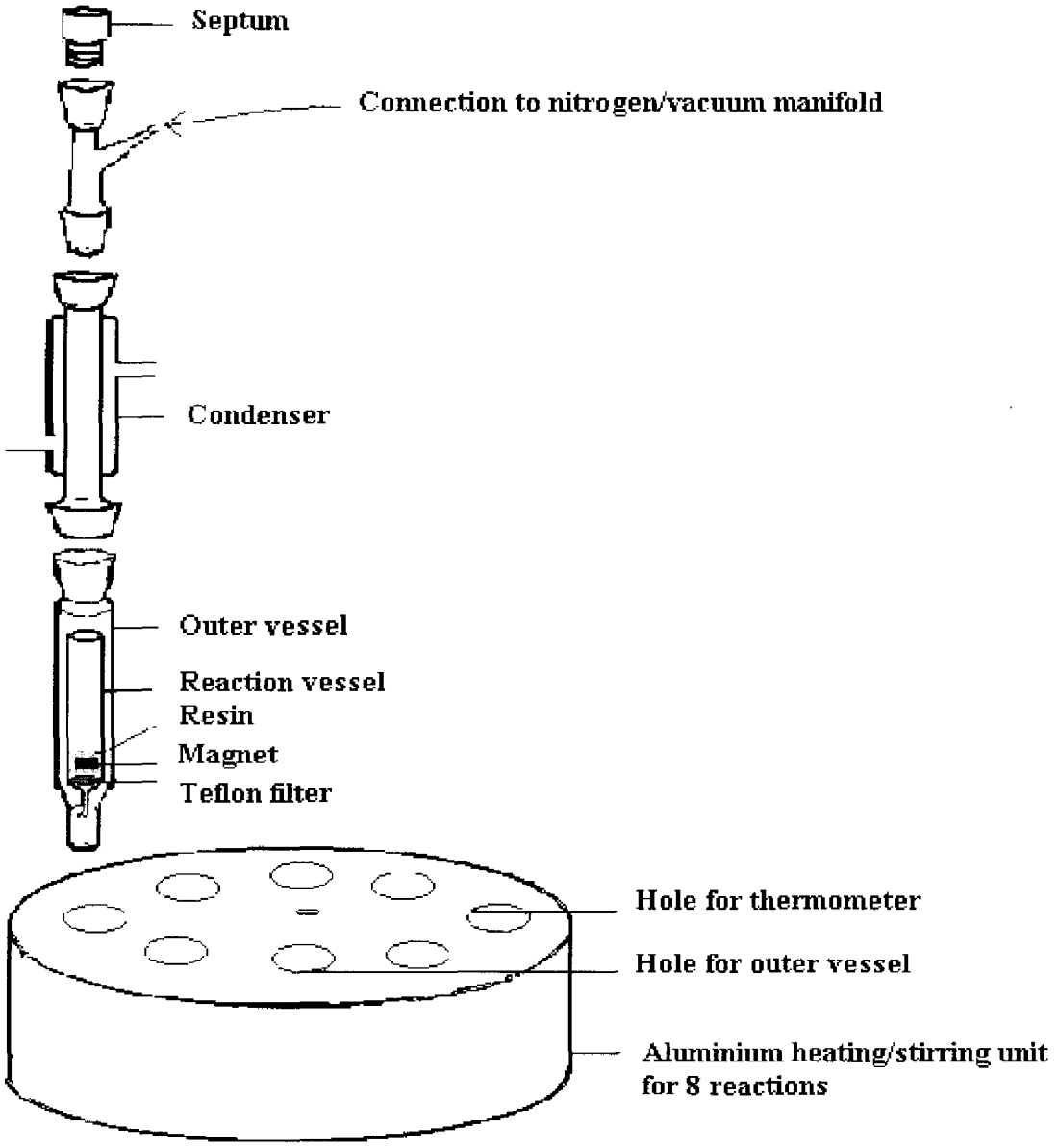


Figure 2. Equipment used for heating/stirring of the solid-phase reactions.

Washing and cleavage

For washing of the resins (in 5 mL reaction vessels) a VacMaster® was used (Figure 3). Reactions vessels (max. 20) were mounted onto the VacMaster and vacuum was applied to remove the residual solvents. The resins were washed with pure solvent to extract impurities from the resin and the solvent was drained as mentioned above. The 20 mL reaction vessels were handled as a regular glass filter. To cleave products from the resin, the resins were treated with TFA. If the resins were treated with TFA for less than 2 hours, the reaction vessel with the resin-bound products were mounted onto the VacMaster® and treated with the appropriate TFA mixture (Figure 3).

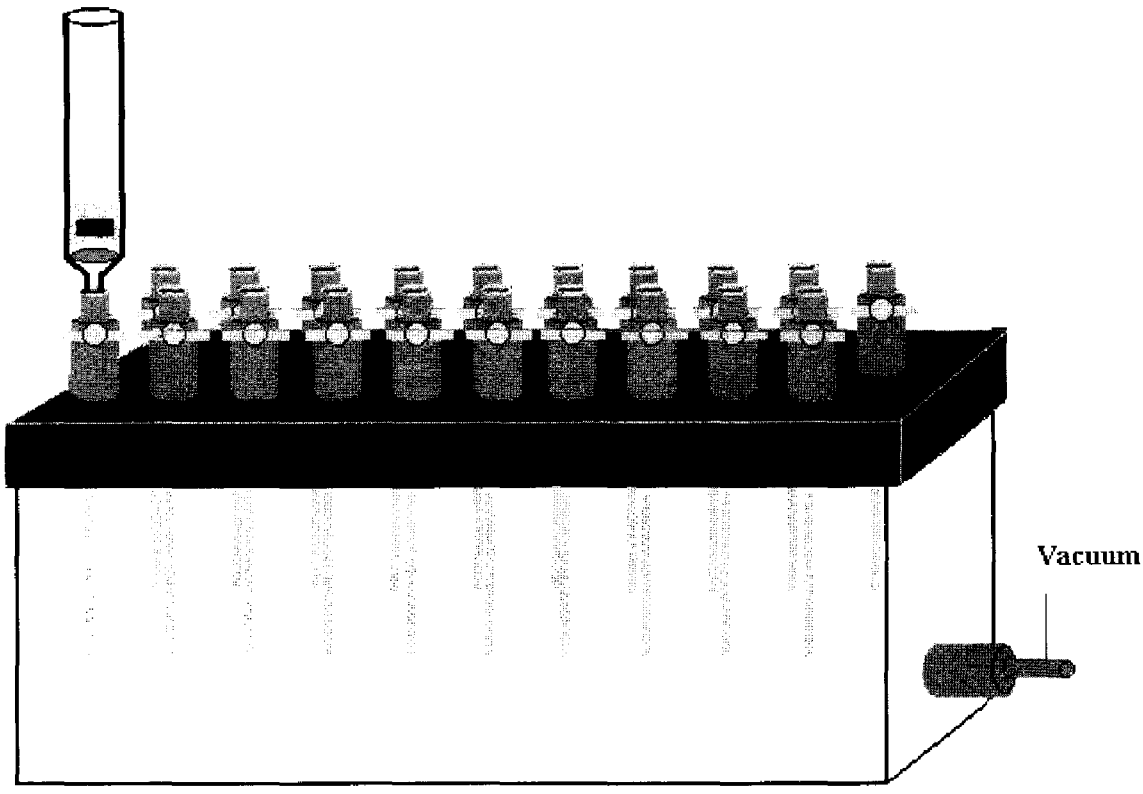


Figure 3. The VacMaster® used for the washing of resins and their subsequent cleavage.

If a longer reaction time was needed to complete the cleavage, the reaction vessels with the resin-bound products inside were placed inside an outer-vessel and treated as any other reaction. If treatment with TFA at reflux was necessary, the heating unit from Figure 2 was used. The mixture resulting from the cleavage was collected in 10 mL glass tubes. These tubes were heated in a heating-unit (max. 20) and a flow of nitrogen was applied to speed-up the evaporation process as depicted in Figure 4.

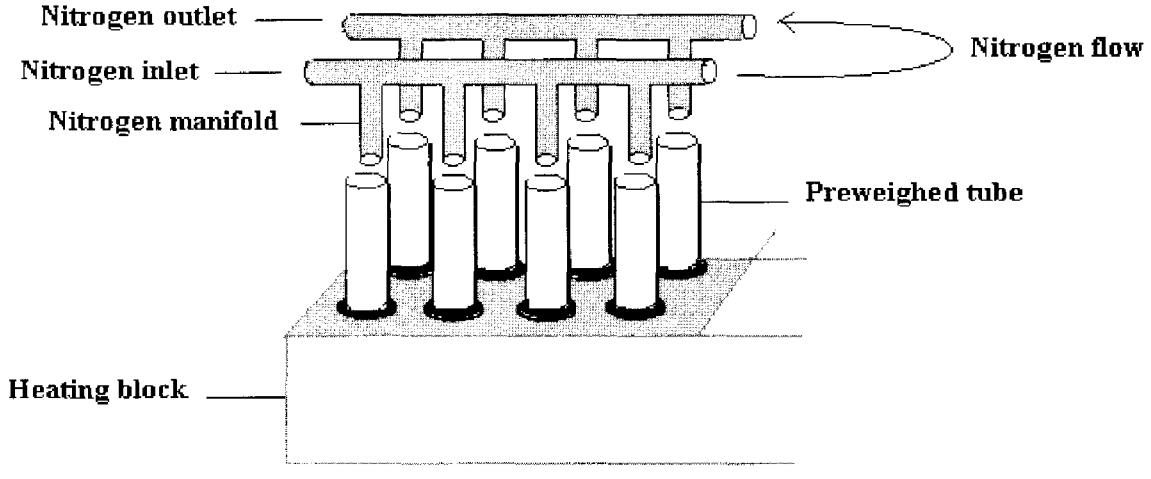


Figure 4. Heating unit with nitrogen manifolds on top used for the evaporation of cleavage mixtures.

Summary

Synthesis and *In Vitro* Pharmacological Evaluation of Novel Ligands for Histamine Receptors

The research described in this thesis involves the study and identification of some of the structural requirements necessary for the displaying of agonism, inverse agonism and neutral antagonism on the human histamine H_1, H_3 and H_4 receptors, by selected classes of ligands. Moreover, in view of the fact that fluorescent ligands combined with confocal microscopy have been proven has a suitable new method for studying receptors and receptor-mediated mechanisms, a part of the research has been dedicated to the search of suitable fluorescent probes to be used as tools for the H_3R.
The distribution, function and agonist-independent signalling of the four known histamine receptors, the reclassification of several known histamine ligands as well as the already known neutral histamine antagonists are reviewed in chapter 1. A brief review on the use of fluorescent ligands for the study of receptors and on the already existent fluorescent ligands for the histamine receptors is given as well.

The aim of this thesis is elucidated in chapter 2: the synthesis and pharmacological evaluation of selected ligands for the histamine H_1, H_3 and H_4 receptors can provide the keys for a better understanding of the molecular features of ligands for these receptors determining their affinity and intrinsic activity. The new ligands should permit to draw structure-activity relationships (SARs) for agonists, inverse agonists and neutral antagonists at the histamine H_1, H_3 and H_4 receptors, ultimately leading to a better understanding of the ligand-receptor interactions and therefore to new insights and ideas for the generation of novel classes of histamine ligands.

In chapter 3 the synthesis and pharmacological identification of neutral antagonists for the histamine H_1 receptor (H_1R) is described. Starting from the endogenous agonist for the H_1R, histamine, various analogues structurally related to H_1R agonists were synthesised and/or tested, which led to the identification of several compounds with partial inverse agonistic properties and two neutral H_1R antagonists (histabudifen and histapendifen). Those compounds may become valuable tools for future pharmacological studies and they may as well serve as starting point for the development of high-affinity neutral H_1R antagonists. The two identified neutral H_1 antagonists exhibit hybrid structures composed of the endogenous agonist histamine combined with the classical H_1R antagonist pharmacophore; consequently,

the modification of agonist structures has proven as a successful strategy to obtain neutral histamine receptor antagonists.

In chapter 4 the synthesis and pharmacological evaluation of imbutamine, impentamine and imhexamine derivatives on the histamine H_3 receptor (H_3R) are reported. In an effort to establish the structural requirements for agonism, neutral antagonism and inverse agonism at the human H_3R a series of higher homologues of histamine in which the terminal nitrogen of the side chain has been either mono- or di-substituted with several aliphatic, alicyclic and aromatic moieties, or incorporated in cyclic systems have been prepared. The novel ligands were pharmacologically investigated *in vitro* for their binding affinities on the human H_3R and H_4R subtypes, and for their intrinsic H_3R activities, and it was recognized that very subtle changes at the substitution in the side chain nitrogen alter enormously the pharmacological activity of the ligands, resulting in a series of compounds spanning the whole spectrum of pharmacological activities. The synthesised series of compounds allowed the identification of the primary amino function of the abovementioned higher homologues of histamine as a chemical switch for the modulation of the functional activity of the compounds on the H_3R.

Chapter 5 reports the synthesis of a series of histamine derivatives bearing a longer and/or unsaturated side chain as well as the corresponding 5-methyl substituted analogues. The synthesised compounds were pharmacologically characterized on the H_3R and H_4R, which led to the identification of (*E*)-4-(5-methyl-1*H*-imidazol-4-yl)-but-3-enylamine and 4-(5-methyl-1*H*-imidazol-4-yl)-butylamine as novel leads for the development of ligands selectively targeting the human H_4R. Interesting SARs for the H_3 and H_4 receptors were derived from the analysed series of ligands, leading to the hypothesis that imbutamine and its derivatives may have a different set of interaction points with the H_3 and H_4 receptors, when compared to impentamine and its derivatives, and a mode of ligand recognition on both receptors similar to the one of histamine.

In chapter 6 the synthesis of targeted imidazole libraries via a solid phase synthesis (SPS) approach is described. Efforts towards a SPS route for the development of imbutamine and impentamine derivatives unfortunately did not lead to the identification of an SPS method that gave substantial advantages in respect to the already used solution phase synthetic pathway for these kind of derivatives. However, the application of a palladium catalysed cross coupling reaction on a solid support for the generation of a library of 22 conformationally constrained analogues of histamine, bearing a triple bond attached directly to the imidazole ring in the 4-position, gave very satisfactory results. The compounds were obtained in moderate to good yields (37-62%) and moderate to excellent purities (37-95%). The synthesised compounds possess a scaffold that can be easily synthesised and modified with a high variety of

substituents via parallel polymer-supported techniques. Evaluation of pharmacological activities of the library compounds on the H_3 and H_4 receptors is reported as well. Moreover, a solution-phase, palladium-catalyzed cross-coupling reaction between an unprotected 4-iodoimidazole and terminal aliphatic and aromatic alkynes, is described for the first time.

In chapter 7 attempts toward the synthesis of potential fluorescent conjugates for the H_3R and their subsequent use as labelling tools are illustrated. Imbutamine and impentamine, two well-known H_3R ligands, were derived with six fluorescent moieties. Some of the synthesised compounds were evaluated for their H_3R affinities and fluorescence properties, as well as for their use as H_3R labelling tools in confocal laser-scanning microscopy experiments. A number of compounds indeed showed good to excellent binding H_3R affinities, but when used as fluorescent probes for the detection of cells expressing the human H_3R, they were found to be unsuitable for the purpose, mainly due to their inadequate physico-chemical properties. Despite our efforts we have so far not been able to synthesise a suitable fluorescent probe for the detection of H_3Rs in living cells. Thus, new synthetic pathways and fluorescent conjugates for the achievement of appropriate fluorescent H_3R ligands will have to be further investigated.

In the appendix the developed glassware and other equipment used for the solid phase synthesis of the imidazole containing compounds reported in chapter 6 are described.

Samenvatting

Synthese en *In Vitro* Farmacologische Karakterisatie van Nieuwe Liganden voor Histamine Receptoren.

Het onderzoek beschreven in dit proefschrift omvat de studie naar en de identificatie van enkele structurele kenmerken van liganden die agonisme, invers agonisme of neutraal antagonisme op de humane histamine H_1, H_3 en H_4 receptoren vertonen. Aangezien de combinatie van fluorescerende liganden met confocale microscopie een nieuwe veelbelovende methode is voor het bestuderen van receptoren en ligand-receptorinteracties is een gedeelte van het onderzoek gewijd aan de ontwikkeling van geschikte fluorescerende verbindingen voor de H_3R.
Een overzicht over de verdeling, de functie en de agonist onafhankelijke signalering van de vier bekende histamine receptoren, tesamen met een reclassificatie van vele bekende histamine liganden en de al bekende neutrale histamine antagonisten wordt in hoofdstuk 1 gegeven. Gevolgd door een kort overzicht over het gebruik van fluorescerende liganden in het onderzoek van receptoren en een overzicht van al bekende fluorescerende liganden voor histamine receptoren.

Het doel van dit proefschrift wordt in hoofdstuk 2 omschreven: de synthese en farmacologische karakterisatie van geselecteerde liganden voor de histamine H_1, H_3 and H_4 receptoren om een beter inzicht in de relatie tussen de moleculaire eigenschappen van liganden en de affiniteit en intrinsieke activiteit voor deze receptoren te verkrijgen. De nieuwe liganden maken het mogelijk om structuur-activiteit relaties (structure-activity relationships, SARs) vast te stellen voor agonisten, inverse agonisten and neutrale antagonisten van de histamine H_1, H_3 en H_4 receptoren. Dit kan tot een beter inzicht in de ligand-receptor interacties leiden en levert ideëen op voor de synthese van nieuwe klassen van histamine liganden.

In hoofdstuk 3 wordt de synthese en farmacologische karakterisatie van neutrale antagonisten voor de histamine H_1 receptor (H_1R) beschreven. Met de endogene agonist, histamine, als startpunt werden diverse analoga gesynthetiseerd en samen met structureel verwante H_1R agonisten getest. Dit leidde tot de identificatie van verschillende verbindingen met gedeeltelijk invers agonistische eigenschappen en twee neutrale H_1R antagonisten (histabudifen en histapendifen). Deze verbindingen vormen waardevolle gereedschappen voor toekomstige farmacologische studies en ze kunnen ook als startpunt voor de ontwikkeling van nieuwe

neutrale H_1R antagonisten met een hoge affiniteit fungeren. De twee geidentificeerde neutrale H_1 antagonisten bezitten een hybride structuur bestaande uit de endogene agonist histamine gecombineerd met de klassieke H_1R antagonist farmacofoor. Modificatie van agonisten blijkt een succesvolle strategie om neutrale antagonisten voor de H_1R te verkrijgen.

In hoofdstuk 4 wordt de synthese en farmacologische karakterisatie van imbutamine, impentamine and imhexamine derivaten voor de histamine H_3 receptor (H_3R) beschreven. Om de structurele vereisten voor agonisme, neutraal antagonisme, en invers agonisme bij de humane H_3R te bepalen werd een serie van langere homologen van histamine gesynthetiseerd en op de eindstandige aminogroep van de zijketen gesubstitueerd met aliphatische, alicyclische, en aromatische groepen, verder werd de aminogroep ook in een ringsysteem opgenomen. De nieuwe liganden werden farmacologisch *in vitro* op hun affiniteiten voor de humane H_3R and H_4R subtypes en voor hun intrinsieke activiteit op de H_3R getest. Uit de resultaten bleek dat kleine veranderingen in de substituent op de aminogroep grote veranderingen in de farmacologische activiteit van de liganden veroorzaakt. De gesynthetiseerde reeks van verbindingen omvat het gehele spectrum van farmacologische activiteiten. De primaire aminogroep van de bovengenoemde langere homologen van histamine fungeert als een chemische schakelaar voor de modulatie van de functionele activiteit van de verbindingen op de H_3R.

In hoofdstuk 5 wordt de synthese van een serie van histamine derivaten met een langere en/of onverzadigde zijketen en al dan niet een 5-methylgroep beschreven. De gesynthetiseerde verbindingen werden op de H_3R en de H_4R farmacologisch gekarakteriseerd. Dit leidde tot de identificatie van (*E*)-4-(5-methyl-1*H*-imidazol-4-yl)-but-3-enylamine en 4-(5-methyl-1*H*-imidazol-4-yl)-butylamine als nieuwe leads voor de ontwikkeling van selectieve liganden voor de humane H_4R. De SARs van de reeks van liganden leidde tot de hypothese dat imbutamine en zijn derivaten een aan histamine vergelijkbare ligand herkenning bezitten en een andere serie van interactiepunten met de H_3R en H_4R receptoren benutten dan de impentamine derivaten.

In hoofdstuk 6 wordt de synthese van een serie imidazool verbindingen via een solid phase synthesis (SPS) benadering beschreven. Pogingen tot ontwikkeling een SPS route voor de synthese van imbutamine en impentamine derivaten leidde niet tot een efficientere methode dan de bestaande vloeistoffase syntheseroute. De toepassing van een palladium-gekatalyseerde cross-couplingreactie in een SPS benadering was wel succesvol en resulteerde in een verzameling van 22 conformationeel starre analoga van histamine met een alkynsubstituent op de 4-positie van de imidazoolring. De verbindingen werden verkregen in matige tot goede opbrengst (37-62%) en een matige tot uitstekende zuiverheid (37-95%). De

gesynthetiseerde verbindingen hebben een basisstructuur die via SPS makkelijk gesynthetiseerd en gemodificeerd kan worden met een grote variëteit aan substituenten. De evaluatie van farmacologische activiteiten van de serie verbindingen op de H_3R en H_4R wordt besproken. Daarnaast worden de eerste succesvolle vloeistoffase, palladium-gecatalyseerde, cross-couplingsreacties tussen een onbeschermd 4-iodoimidazool en aliphatische en aromatische alkynen beschreven.

In hoofdstuk 7 worden de pogingen tot de synthese van potentiële fluorescerende verbindingen voor de H_3R en hun gebruik als label beschreven. Imbutamine en impentamine, twee bekende H_3R liganden, werden gemodificeerd met zes verschillende fluorescerende substituenten. Enkele van de gesynthetiseerde verbindingen werden getest op hun H_3R affiniteit, fluorescerende eigenschappen, en als fluorescentielabel in experimenten met een confocale laser-scanning microscope. Een aantal verbindingen vertoonde een goede tot uitstekende affiniteit voor de H_3R, maar waren vanwege hun ontoereikende fysische en chemische eigenschappen niet geschikt als fluorescentielabel voor de detectie van levende cellen met de humane H_3R's. Voor de verdere ontwikkeling van geschikte labels zullen nieuwe syntheseroutes en fluorescente groepen bekeken moeten worden.

In de bijlage wordt het glaswerk en de uitrusting beschreven die voor de SPS van imidazool bevattende verbindingen in hoofdstuk 6 is gebruikt.

Acknowledgments

I would like to start this section by expressing my deepest gratitude to my family. Without them this Ph.D. thesis would not exist. First of all my parents, Aldina and Sergio. Cari mamma e papà, grazie di cuore per tutto quello che avete fatto per me in tutti questi anni, soprattutto quelli passati all'estero, e per avermi sostenuta (e sopportata!) nei momenti di sconforto. A big thank goes also to my brother, Riccardo. Caro Ricky, grazie per aver contribuito, assieme a mamma e papà, a farmi sentire sempre come se fossi a casa assieme a voi, ed anche un enorme grazie per il tuo preziosissimo supporto IT!

I am indebted to my promoters, Prof. Dr. Henk Timmerman and Prof. Dr. Rob Leurs, for giving me the opportunity of carrying out this Ph.D. project in Amsterdam. Dear Henk and Rob, thank you for your enthusiasm and support for my work. I express my gratitude also to Dr. Wiro Menge, who has guided me during the first two years of my Ph.D. project. Dear Wiro, thank you very much for the practical help in the lab, the fruitful discussions and creative suggestions. A huge thank especially for the quick review of my samenvatting! Thanks also to Dr. Iwan De Esch, who followed my work in the last months of my Ph.D. project. Dear Iwan, thank you for your help and support!

My gratitude goes also to Prof. Dr. Carlo Melchiorre and Prof. Dr. Piero Valenti, who told me about the available Ph.D. position in Amsterdam and encouraged me to apply for it.

I want to express my gratitude to all the (many!) colleagues I met during the years of my Ph.D. for creating a stimulating and friendly working environment, but also for the leisure moments during the coffee breaks. Among them, Stefania (who is a friend before being a colleague), Ruengwit, Andrea, Sarel, Janneke, Edith, and many others!

All the members of the School of Pharmaceutical Sciences and Institute of Cell Signalling of the University of Nottingham are gratefully acknowledged. A special thank to Prof. Dr. Steve Hill and Dr. Barrie Kellam for all the guidance, fruitful discussions and helpful suggestions. A big thank also to the other members of the group, especially Richard, Monica, Andy, Mike and Paul, for the fun we had in the lab and outside!

Aside from work, I have wonderful memories of my experience in Amsterdam due to my dearest Italian (or Italian ad honorem) friends. The complete list would be too long, but a big big big thank you goes to (in random order!), Stefania, Diego, Alois, Elena and Sergio, Ludovica, Marina and Luca, Calina, Daniele (a special word of thank for the Dutch translation of the summary!), Alessandro, Maria Grazia, Vittorio, Stefano, Wolf, Gaia, Roberto, Chiara and Alberto, Alan and Veronica, Luisa and Yves and many others. Thanks to all of you for making my life in Amsterdam different! Un grazie speciale agli amici più intimi che hanno diviso i momenti di gioia e di sconforto di questi anni e mi hanno fatto sentire a casa lontano da casa.

I also would like to thank my ex-neighbor and good friend Jorge, who helped me a lot at the beginning of my "Dutch adventure". Dear Jorge, thanks for showing me the wonders of Amsterdam nightlife and for introducing me to all your Spanish friends! Thanks also to all the Spanish emigrants who welcomed me in their group and made my first months in a foreign country much better!

Speaking of first months in a foreign country, many thanks to Judith and Enrico, for providing the accomodation and the priceless help with the practical problems at the beginning of my stay in The Netherlands.

A special thought goes to all my friends in Italy, who always welcomed me every time I went back or came to visit me in The Netherlands. Again, the complete list would be too long, but many thanks to Marcy, Andrea, Mirco, Maurizio, Tony, Stefano, Alessandro, Francesco and all the rest of the gang!

Finally I would like to conclude by thanking all those who, due to a failure in my memory, I might have forgotten to mention here but still contributed in various ways to the completion, noy only of this thesis, but of this stage of my life.

It has been a great experience, thank to you all!!